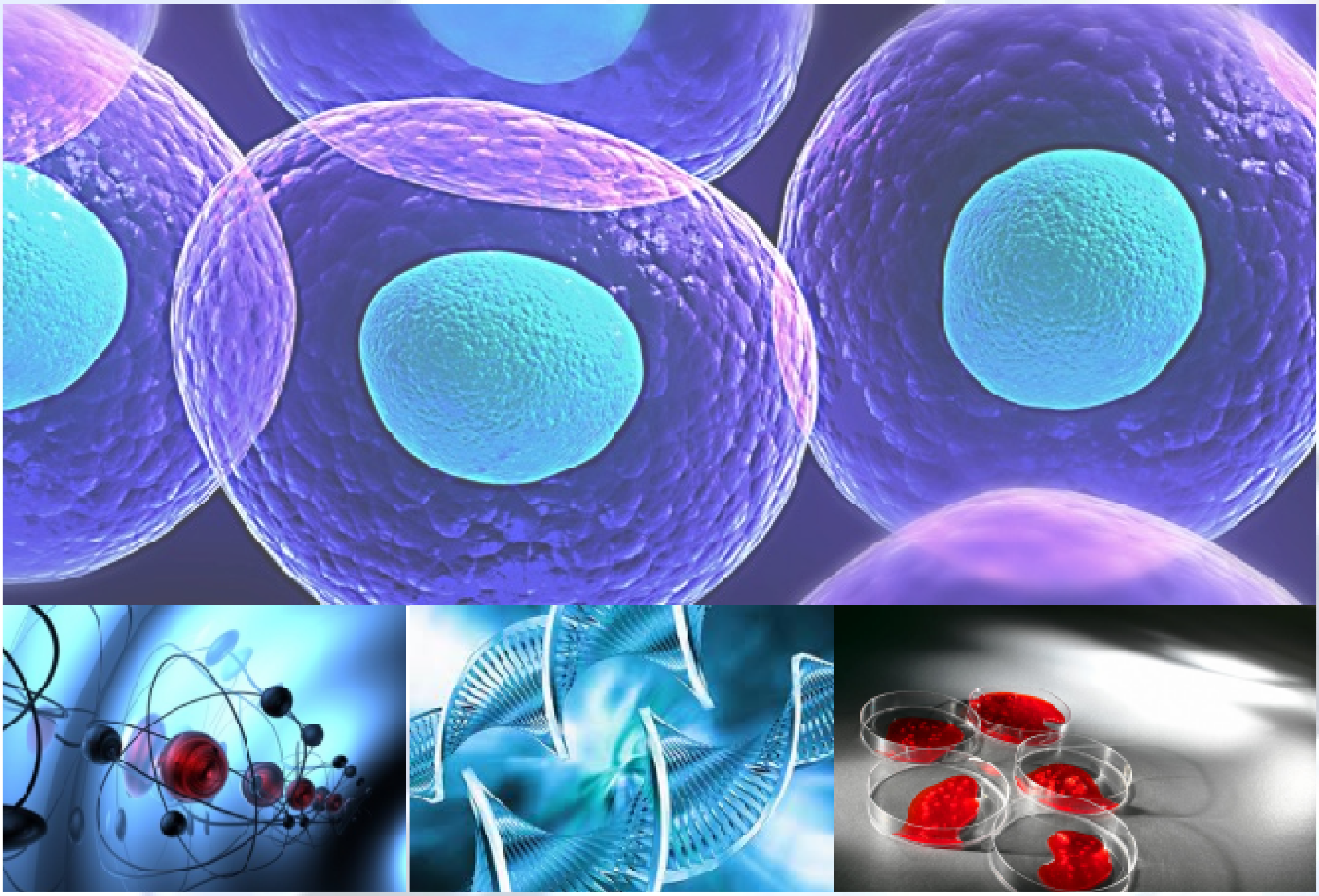
CURRENT TRENDS IN ADVANCEMENT OF SCIENTIFIC RESEARCH AND OPINION IN APPLIED MICROBIOLOGY AND BIOTECHNOLOGY



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SCIENCE AND EDUCATION DEVELOPMENT INSTITUTE, NIGERIA

CURRENT TRENDS IN ADVANCEMENT OF SCIENTIFIC RESEARCH AND OPINION IN APPLIED MICROBIOLOGY AND BIOTECHNOLOGY: ***AN INTRODUCTION TO READERS***

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PREFACE

The book provides an insight and detailed knowledge on the various aspects of applied microbiology and biotechnology as is going on in the present research trend. The book discusses on various integral issues related to microbiology and biotechnology which will prove to be of immense help to the academicians, scientists, researchers, technocrats and students worldwide. The book is a sincere work of compilation of new and recent advances in the topic of concern through various innovative researches and scientific opinion there from. The book is dedicated to the readers who will definitely find it interesting and knowledgeable in carrying out their respective researches in different aspects of applied microbiology and biotechnology.

Lastly, the Science and Education Development Institute [SEDInst], Akure, Nigeria is coming up with more Book Projects very soon on other disciplines currently in demand for the advancement of science, education and technology.

CHAPTER 1

INTRODUCTION

CURRENT TRENDS IN ADVANCEMENT OF SCIENTIFIC RESEARCH AND OPINION IN APPLIED MICROBIOLOGY AND BIOTECHNOLOGY: *AN INTRODUCTION TO READERS*

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Lactic acid bacteria are important as natural biopreservatives which possess antagonistic properties against the spoilage bacteria and pathogens. The metabolites of LAB include acidic components like acetic acid, lactic acid, hydrogen peroxide and bacteriocins which are peptide in nature. When the LAB competes for nutrients then they produce these metabolite components and the antimicrobial product, nisin which acts as a promising preservative for food.

The bacterciocins produced by LAB are also used in the Hurdle Technology. Using the bacteriocin producing LAB with other effective preservation techniques is effective in controlling the growth of spoilage microorganisms and inhibiting their generation, growth and bioactivity.

Biopreservation is the method used for food preservation by using natural antimicrobials and microbiota thereby increasing the storage life of food. The beneficial products formed due to fermentation by the bacteria are used in this process to reduce the rate of food spoilage and to render the food free from pathogenic microorganisms and metabolites. This process is gradually increasing in popularity for its ecologically benign approach.

Hurdle technology is another method of rendering the food to be free from contaminating and spoilage bacteria and pathogens by the combination of one or more methods. The pathogenic microorganisms have to pass through these individual approaches called "hurdles" for maintaining their activity in food products. Proper combination of hurdles will lead to destruction of the microbes and can prevent their further growth. The proper combination of the hurdles ensures the microbial safety in food thereby maintaining its nutritional and organoleptic parameters for consumer preference. The hurdles include the properties like processing at high temperature, storage at low temperature, lowering pH (increasing acidity), water activity (a_w) and/ or redox potential including the presence of biopreservatives or other preservative components in food products. The intensity of the hurdle is ascertained and controlled according to the type of spoilage microorganism(s) and regulated as per consumer safety and preference without sacrificing the quality and appearance of the final food product.

CHAPTER 2
NATURALLY DERIVED SUBSTANCES HAVING ANTIMICROBIAL PROPERTIES FOR EXTENDING STORAGE LIFE OF FOOD

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ABSTRACT

Chitosan is polysaccharide in nature with linearity in arrangement and composed of β-(1-4)-linked D-glucosamine (deacetylatedunit)and N-acetyl-D glucosamine (acetylated unit) which remain in a random distribution. Chitin and the derived chitosan having promising antimicrobial properties, both have potential industrial and biomedical implications for treatment of many ailments in human beings.

KEY WORDS: Biomedical, Chitosan

INTRODUCTION

The shrimp waste contains biopolymers chitin, chitosan, protein with high economical values. Chitosan [ß-(1→4)-linked *N*-acetyl-D-glucosamine] which is the major structural component of the exoskeleton of crustacean is a non-toxic, biodegradable polymer of high molecular weight, and is very much similar to cellulose. In South-east Asia, the total waste produced is over 2 million metric tonnes per year. (Hossain, 2003). Production cost for 1 kg of chitosan is about US$ 15-20/kg (Stevens *et al*., 1996). 12 kg better quality chitosan is obtained from 200 kg shrimp bio waste (Stevens *et al*., 1998).

Chitosan is a new promising technology developed to kill and inactivate undesirable microorganisms in more environmental friendly way without affecting food quality. Shellfish chitosan from crab and shrimp comprises of 17-32% of the dry weight of the shell. (No and Meyers, 1992) Chitosan emerged from chitin, which is available commercially and used by a lot of people across the globe. Many studies have shown that chitosan is biodegradable polymer (Davies *et al*., 1989). Chitosan is a biomaterial with antiseptic, bioactive, and biocompatible properties (Shigemasa *et al.,* 1994). Chitosan has been approved as functional food in some south-east Asian countries during the last decade. The inclusion of chitin and chitosan was considered in 2003 by the Codex Alimentarius Commission.

Chitosan coating helps to extend shelf life of marine and seafood by reducing microbial load and respiration rate, act as antioxidant and oxygen barrier. The performance of the films will be influenced by the acetic acid (used to soluble chitosan) also. Suitability of the film will also depend on the conditions of storage, namely the temperature and humidity (Bhadra *et al*., 2012).

Sea food industry suffers from different problems like post-processing contamination by bacteria like *Escherichia coli* and *Staphylococcus aureus* during retail display and handling, Lipid oxidation due to contact with oxygen. The above problems shows the way for future research on the use of chitosan as coating film to improve shelf life of products like PUD shrimps, fillets etc that are kept on retail display (Bhadra *et al*., 2012).

Keeping this in view, Bhadra *et al*. (2012) performed the study to determine the proximate composition of shrimp waste, to extract chitosan from shrimp waste for determining its antimicrobial activity on *Staphylococccus aureus* and *E. coli* to assess the storage life of *P. monodon* coated with chitosan film.

Chitosan for use in industrial purpose is extracted from the chitin after deacetylation of chitin which is the major structural element of the exoskeletons of crustaceans and lobsters, prawns and cell wall of fungi.

Wide range of applications

Chitosan finds its application in water treatment plants as a part in the filtration process. It helps in the removal of hardness by the removal of sediments during salt filtration by adsorption of phosphorus, heavy minerals and oils from the water in combination with bentonite, gelatin, silica gel, isinglass or other refining agents. Chitosan also helps in clearing the turbidity in water and also used as a precipitant for casein used for manufacturing cheese from milk.

Biomedical importance

Chitosan has the property to clot blood and so also used in haemostatic agents for acceleration of wound healing property in humans. Chitosan also possess hypoallergenic and has natural antibacterial properties, which further support its use in field bandages. The flexible nature of chitin is used for making strong and tensile surgical threads having high biodegradability and wound healing property.The antimicrobial property of chitosan is attributed to its slightly acidic pH (Bhadra *et al.*, 2012).

Chotosan in ultrapure form has numerous biomedical implications. Chitosan has mucoadhesive property and so used for intradermal and sustained drug delivery (Agnihotri *et al.*, 2004), for example, in the administration of insulin in insulin dependent diabetes mellitus. Chitosan is biocompatible and biodegradable which enhances the polar drug transport across epithelial membranes and surfaces (Baldrick, 2009).

Proven research fact

Chitosan coating serve as an antioxidant and micro-diffusion barrier and prevents the loss of water, texture, odor and color thereby improving the overall accessibility of the seafood. The shelf life of *Penaeus monodon* coated with chitosan dips extends the shelf life of shrimp (Bhadra *et al.*, 2012).

Chitosan also helps in increased lipid excretion from the body system after interaction and binding with oily components in the digestive tract. Its fat binding property helps in decrease of body mass index especially in obese patients who are advised to be supplemented with the chitosan for nearly 8 weeks continuously for encouraging results. Chitosan possesses the functional antioxidant, antimicrobial and oxygen barrier properties (Fan *et al.*, 2008). No *et al.* (2002) reported that 1% chitosan concentration can retard gram negative bacteria like *E. coli*, *Vibrio, Salmonella* etc. The effect of chitosan may be due to the change in the outer membrane of *E. coli* cell, thereby affecting the barrier properties of bacterial cell (Helander *et al.*, 2001). Sudarshan *et al.* (1992) reported that leakage of intracellular material was one of the mechanisms of chitosan actions which mainly signify the present study. No *et al.* (2002) reported the use of chitosan is effective against Gram-positive bacteria like *Staphylococcus aureus*. Muzzarelli *et al.* (1990) reported the effect of chitosan and its derivatives in inhibiting *Staphylococcus aureus* and other gram-positive

bacteria. The antibacterial mechanism of chitosan may be attributed to the interaction of charged amino group of chitosan with negatively charged microbial cell membrane leading to the leakage of proteinaceous and other intracellular constituents of the microorganisms. (Shahidi et *al*., 1999).

Seafood provides a good niche for growth of different microorganisms. The initial total plate count of fresh *P. monodon* samples were 3.02 log CFU/g. Chitosan coatings resulted in upto 2-3 log reductions in total plate count between coated samples and control after 12 days of storage of herring and cod in refrigerated storage condition. Chitosan coating significantly lowered the TPC in fish samples ($P<0.05$) with 0.60-1.19 log CFU/g reduction being obtained in coated samples and the TPC of coated samples were below 10^7 CFU/g during first 2 weeks of cold storage. In the present study chitosan coatings resulted in 3-4.5 log CFU/g reduction in total plate count at $4^{o}\pm1^{o}C$ storage temperature as compared to uncoated one (Bhadra *et al.,* 2012).

Chitosan coating consisting of a blend of acetic acid and 1% chitosan exerts an inhibitory effect on the gram- negative flora of fish patties. Various factors affect the antimicrobial action of chitosan and its mechanism of action appears to be related to interactions between the positively charged chitosan molecules and the negatively charged microbial cell membrane and act as a barrier against oxygen transfer. From the results of this study, it was concluded that chitosan coating has a significant ($P<0.05$) advantage in retarding the growth of bacteria. From the result of the study indicated that 1% chitosan solution coating was effective as 2% chitosan coated samples for extending the shelf life at $4^{o}\pm1^{o}C$storage temperature, attributing to the better inhibitory effect of 1% chitosan on spoilage bacteria (Bhadra *et al.,* 2012).

Antimicrobial properties

Low molecular weight chitosan seemed to be a more effective inhibitor of microbial growth for some organisms such as *E. coli*. Although chitosan generally show stronger bactericidal effect on gram positive bacteria, in the present study it is found to be effective in inhibiting the gram negative bacteria *E .coli* at a concentration of both 1% and 2% level. The effect of chitosan may be due to the change in the outer membrane of *E. coli* cell, thereby affecting the barrier properties of bacterial cell. Leakage of intracellular material was one of the mechanisms of chitosan actions which mainly signify the present study (Bhadra *et al.,* 2012).

The use of chitosan is effective against Gram-positive bacteria like *Staphylococcus aureus*. Effect of chitosan and its derivatives is shown in inhibiting *Staphylococcus aureus* and other Gram-positive bacteria. The antibacterial mechanism of chitosan may be attributed to the interaction of charged amino group of chitosan with negatively charged microbial cell membrane leading to the leakage of proteinaceous and other intracellular constituentents of the microorganisms (Bhadra *et al.,* 2012).

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CHAPTER 3
BIOSYNTHESIS AND CHARACTERIZATION OF NANOPARTICLES

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What is Nanotechnology?
Nanotechnology is the study of manipulating matter on an atomic and molecular scale. Generally, nanotechnology deals with particles sizes between 1 and 100 nanometer in at least one dimension. Quantum mechanical effects are very important at this scale, which is in the quantum realm. Nanotechnology is very diverse, ranging from extensions of conventional device physics to completely new approaches based upon molecular self-assembly, from developing new materials with dimensions on the nanoscale to investigating whether we can directly control matter on the atomic scale. The potential of nanotechnology to revolutionize the health care, textile, materials, information and communication technology, and energy sectors has been well-publicized. In fact several products enabled by nanotechnology are already in the market, such as antibacterial dressings, transparent sunscreen lotions, stain-resistant fabrics, scratch free paints for cars, and self cleaning windows. The application of nanotechnology to the agricultural and food industries was first addressed by a United States Department of Agriculture roadmap published in September 2003. The prediction is that nanotechnology will transform the entire food industry, changing the way food is produced, processed, packaged, transported, and consumed.

The definition of nanotechnology is based on the prefix "nano" which is from the Greek word meaning "dwarf". In more technical terms, the word "nano" means 10-9, or one billionth of something. For comparison, a virus is roughly 100 nanometres (nm) in size. The word nanotechnology is generally used when referring to materials with the size of 0.1 to 100 nanometres, however it is also inherent that these materials should display different properties from bulk (or micrometric and larger) materials as a result of their size. These differences include physical strength, chemical reactivity, electrical conductance, magnetism, and optical effects.

Nanotechnology may be able to create many new materials and devices with a vast range of applications, such as in medicine, electronics, biomaterials and energy production. On the other hand, nanotechnology raises many of the same issues as any new technology, including concerns about the toxicity and environmental impact of nanomaterials, and their potential effects on global economics, as well as speculation about various doomsday scenarios. These concerns have led to a debate among advocacy groups and governments on whether special regulation of nanotechnology is warranted.

Origin: History of nanotechnology
The first use of the concepts found in 'nano-technology' (but pre-dating use of that name) was in "There's Plenty of Room at the Bottom", a talk given by physicist Richard Feynman at an American Physical Society meeting at California Institute of Technology (Caltech) on December 29, 1959. Feynman described a process by which the ability to manipulate individual atoms and molecules might be developed, using one set of precise tools to build and operate another proportionally smaller set, and so on down to the needed scale. In the course of this, he noted, scaling issues would arise from the changing magnitude of various physical phenomena: gravity would become less important, surface tension and Vander

Waal attraction would become increasingly more significant, etc. This basic idea appeared plausible, and exponential assembly enhances it with parallelism to produce a useful quantity of end products. The term "nanotechnology" was defined by Tokyo University of Science Professor Norio Taniguchi in a 1974 paper as follows: "'Nano-technology' mainly consists of the processing of, separation, consolidation, and deformation of materials by one atom or by one molecule." In the 1980s the basic idea of this definition was explored in much more depth by Dr. K. Eric Drexler, who promoted the technological significance of nano-scale phenomena and devices through speeches and the books Engines of Creation: The Coming Era of Nanotechnology (1986) and Nanosystems: Molecular Machinery, Manufacturing, and Computation, and so the term acquired its current sense. The "Engines of Creation" is considered the first book on the topic of nanotechnology. Nanotechnology and nanoscience got started in the early 1980s with two major developments; the birth of cluster science and the invention of the scanning tunneling microscope (STM). This development led to the discovery of fullerenes in 1985 and carbon nanotubes a few years later. In another development, the synthesis and properties of semiconductor nanocrystals was studied; this led to a fast increasing number of metal and metal oxide nanoparticles and quantum dots. The atomic force microscope (AFM or SFM) was invented six years after the STM was invented.

Fundamental concepts

Nanotechnology is the engineering of functional systems at the molecular scale. This covers both current work and concepts that are more advanced. In its original sense, nanotechnology refers to the projected ability to construct items from the bottom up, using techniques and tools being developed today to make complete, high performance products. One nanometer (nm) is one billionth, or 10^{-9} meter. By comparison, typical carbon-carbon bond lengths, or the spacing between these atoms in a molecule, are in the range 0.12–0.15 nm, and a DNA double-helix has a diameter around 2 nm. On the other hand, the smallest cellular life-forms, the bacteria of the genus mycoplasma, are around 200 nm in length. By convention, nanotechnology is taken as the scale range 1 to 100 nm following the definition used by the National Nanotechnology Initiative in the US. The lower limit is set by the size of atoms (hydrogen has the smallest atoms, which are approximately a quarter of a nm diameter) since nanotechnology must build its devices from atoms and molecules. The upper limit is more or less arbitrary but is around the size that phenomena not observed in larger structures start to become apparent and can be made use of in the nano device. These new phenomena make nanotechnology distinct from devices which are merely miniaturized versions of an equivalent macroscopic device; such devices are on a larger scale and come under the description of micro technology.

Why Nanotechnology Approach-

Nanotechnology approach is used because of the following reason-

- High surface area and high reactivity
- Effective catalyst of plant/microbial metabolism
- Better penetration into the cell
- Increased both plant and microbial activities
- Nanotechnology opened doors to new ways of identifying and quantifying biomolecules through use of nanosensors and nanoprobes
- Nanotechnology offers the tools to understand and transform biosystems; strong impact on sub-cellular dynamics; regeneration mechanisms; genome description; food characterization

- A new platform for synthesis of effective chemicals and biodegradable, food preparation and conservation; sensors and control.

Synthesis of nanoparticles
A series of general methods for the nanoparticle synthesis have now been developed. An essential feature of their synthesis is the preparation of particles of specified size and shape (at least, the scatter of sizes should be small, 5% ± 10%, and controllable). The shape control and the possibility of synthesis of anisotropic structures are especially important. There are two main approaches are used in nanotechnology- bottom-up and top down approach. In the "bottom-up" approach, materials and devices are built from molecular components which assemble themselves chemically by principles of molecular recognition. In the "top-down" approach, nano-objects are constructed from larger entities without atomic-level control.

The definition of nanotechnology does not include unintentionally produced nanomaterials, such as diesel exhaust particles or other friction or airborne combustion byproducts, or nanosized materials that occur naturally in the environment, such as viruses or volcanic ash. Information from incidentally formed or natural nanosized materials (such as ultrafine particulate matter) may aid in the understanding of intentionally produced nanomaterials. Intentionally produced nanomaterials are catagorised as Carbon-based (hollow spheres, ellipsoids, and nanotubes), Metal-based (quantum dots, nanogold, nanosilver and metal oxides), Dendrimers (nanosized polymers) and Composites nanomaterials. The unique properties of these various types of intentionally produced nanomaterials give them novel electrical, catalytic, magnetic, mechanical, thermal, or imaging features that are highly desirable for applications in commercial, agricultural, medical, military, and environmental sectors. Nanoparticles are viewed as the fundamental building blocks of nanotechnology. They are the starting points for preparing many nanostructured materials and devices. Their synthesis is an important component of the rapidly growing research efforts in nanoscience and nanoengineering.

Synthesis of nanoparticles with a range of compositions, sizes and shapes has been demonstrated by various physical, chemical and biological means. Some of the very successful physical and chemical methods for the synthesis of nanoparticles include laser ablation, ion sputtering, solvothermal synthesis, chemical reduction, sol-gel, photo-irradiations, radiolysis, ultrasonication, spray pyrolysis, solvated metal atom dispersion, chemical vaporization, and electrochemical methods.

Drawback of “physical” approaches is enormous consumption of energy to maintain the high pressure and temperature used in the synthesis procedures. The traditional and most widely used methods for synthesis of metallic nanoparticles use wet-chemical procedures. A typical procedure involves growing nanoparticles in a liquid medium containing various reactants, in particular reducing agents e.g., sodium borohydride or potassium bitartrate or methoxy poly ethylene glycol and hydrazine. To prevent the agglomeration of metallic nanoparticles, a stabilizing agent such as sodium dodecyl benzyl sulfate or polyvinyl pyrrolidone is also added to the reaction mixture. Generally, the chemical methods are low-cost for high volume. However, their drawbacks include contamination from precursor chemicals, use of toxic solvents, and generation of hazardous by-products.

Hence, there is an increasing need to develop high-yield, low cost, nontoxic, different chemical compositions, sizes, controlled monodispersity and environmentally benign

procedures for synthesis of metallic nanoparticles. As a result, researchers in the field of nanoparticle synthesis and assembly have turned to biological systems for inspiration. This is not surprising that many organisms, both unicellular and multicellular, are known to produce inorganic materials either intra or extra cellularly. A vast array of biological resources available in nature including plants and plant products, algae, fungi, yeast, bacteria, and viruses could be employed for synthesis of nanoparticles.

It is well known that many organisms can provide inorganic materials either intra or extra cellularly. For example, unicellular organisms such as magnetotactic bacteria produce magnetite nanoparticles, and diatoms synthesize siliceous materials. Multicellular organisms produce hard inorganic-organic composite materials such as bones, shells, and spicules using inorganic materials to build a complex structure. These biominerals are composite materials and consist of an inorganic component and a special organic matrix i.e. proteins, lipids, or polysaccharides that controls the morphology of the inorganic compound. The surface layer bacteria produce gypsum and calcium carbonate layers. Even though many biotechnological applications such as the remediation of toxic metals employ microorganisms such as bacteria and fungi, such microorganisms are act as eco-friendly nanofactories. Processes devised by nature for the synthesis of inorganic materials on nano and micro length scales have contributed to the development of a relatively new and largely unexplored area of research based on the use of microbes in the biosynthesis of nanomaterials.

The use of fungi in the synthesis of nanoparticles is a relatively recent addition to the list of microorganisms. The use of fungi is potentially exciting since they secrete large amounts of enzymes and are simpler to deal with in the laboratory. However, the genetic manipulation of eukaryotic organisms as a means of overexpressing specific enzymes identified in nanomaterial synthesis would be much more difficult than that in prokaryotes. In addition to good monodispersity, nanoparticles with well defined dimensions can be obtained by using fungi. Bioreduction of aqueous $AuCl_4^-$ ions was carried out using the fungus *Verticillium* sp. that led to the formation of gold nanoparticles with fairly well-defined dimensions and good monodispersity. These results have been documented that the trapping of $AuCl_4^-$ ions on the surface of fungal hyphae could occurs by electrostatic interaction with positively charged groups i.e. lysine residues in enzymes that are present in the cell wall of the mycelia. In this enzyme mediated reaction, gold ions were reduced by enzymes within the cell wall leading to aggregation of metal atoms and formation of gold nanoparticles. However, they could not find the exact mechanism of formation of gold nanoparticles. It can be concluded from their study that bacteria, fungi could be a source for large scale production of nanoparticles.

Since fungi are known to secrete much higher amounts of proteins, thus might have significantly higher productivity of nanoparticles in biosynthetic approach. Towards elucidating mechanism of nanoparticles formation, an *in vitro* approach was followed, where, species specific NADH dependent reductase, released by the *Fusarium oxysporum*, were successfully used to carry out the reduction of $AuCl_4^-$ ions to gold nanoparticles. This has first time opened up a novel fungal/enzyme-based *in vitro* approach for nanomaterials synthesis. Based on properties of *Fusarium oxysporum*, it was also used in the formation of extremely stable silver hydrosol. The acidophilic fungus *Verticillium* sp. has capability of producing gold as well as silver nanoparticles upon their incubation with Ag^+ and $AuCl_4^-$ ions. However, a novel biological method for the intra and extra cellular synthesis of silver nanoparticles using the fungi, *Verticillium* sp. and *Fusarium oxysporum* respectively has

been documented. This has opened up an exciting possibility, wherein, the nanoparticles may be entrapped in the biomass in the form of a film or produced in solution, both having interesting commercial potentials. The fungus, *Aspergillus flavus* also resulted in the accumulation of silver nanoparticles on the surface of its cell wall, when incubated with silver nitrate solution.

Extracellularly produced nanoparticles were stabilized by the proteins and reducing agents secreted by the fungus. Four high molecular weight proteins released by the fungal biomass have been found in association with nanoparticles. Further, the reduction of metal ions and surface binding of the proteins to the nanoparticles did not compromise the tertiary structure of the proteins. Biomolecules likes polypeptide and enzymes carry out reduction reaction to synthesize gold nanoparticles by endophytic fungus *Collitotrichum* species. Instead of fungi culture, isolated proteins from fungi have been successfully used for nanoparticles production.

Growth conditions play an important role during the production of nanoparticles while using the fungi cultures. During gold nanoparticle synthesis, it was observed that when gold ions were incubated with the *Trichothecium* sp. biomass under stationary conditions led to the formation of extracellular nanoparticles, whereas under shaking conditions, this was resulted in the formation of intracellular gold nanoparticles. These proteins were released into the medium under stationary conditions and did not release under shaking conditions. Both fungi secreted proteins which were capable of hydrolyzing iron precursors extracellularly to form iron oxides predominantly in the magnetite (Fe_3O_4) phase. This was not true with all *Fusarium* sp., *Fusariummoniliforme* produces reductase enzyme but could not form silver nanoparticles upon their incubation with silver ions. However, by controlling the amount of cofactor NADH, synthesis of quite stable Au–Ag alloy nanoparticles of various compositions have been produced. This approach can be further employed for producing various other composite nanoparticles. Towards elucidating the mechanism of synthesis of nanoparticles, NADPH-dependent nitrate reductase and phytochelatin isolated from *Fusarium oxysporum* has been used for *in vitro* silver nanoparticle production. The production of nanoparticle through biological route is easy and even faster as compared to the physical and chemical processes of nanoparticles synthesis. A general flow chart for nanoparticle biosynthesis is given in Figure 1.

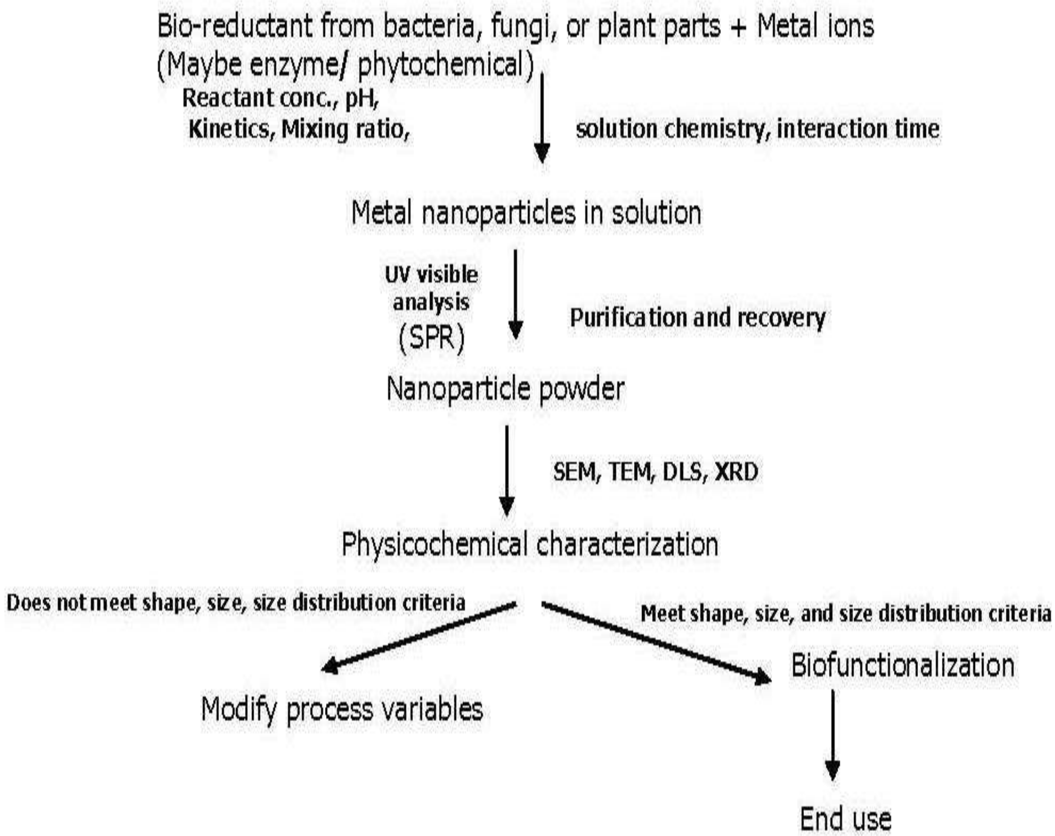

Figure 1 - A basic approach towards biosynthesis of nanoparticles

Characterization of biosynthesized nanoparticles

Nanoparticle characterization is necessary to establish understanding and control of nanoparticle synthesis and applications. Characterization is done by using a variety of different techniques, mainly drawn from materials science. Common techniques are electron microscopy (TEM, SEM), atomic force microscopy (AFM), dynamic light scattering (DLS), x-ray photoelectron spectroscopy (XPS), powder X-ray diffraction (XRD), Fourier transform infrared spectroscopy (FTIR), matrix-assisted laser desorption-ionization time-of-flight mass spectrometry (MALDI-TOF), ultraviolet-visible spectroscopy, dual polarization interferometer and nuclear magnetic resonance (NMR).

DLS analysis

Dynamic light scattering also known as photon correlation spectroscopy or quasi elastic light scattering is a technique in physics, which can be used to determine the size distribution profile of small particles in suspension or polymers in solution. It can also be used to probe the behavior of complex fluids such as concentrated polymer solutions. When light hits small particles the light scatters in all directions (Rayleigh scattering) so long as the particles are small compared to the wavelength (below 250 nm). If the light source is a laser, and thus is monochromatic and coherent, then one observes a time-dependent fluctuation in the scattering intensity. These fluctuations are due to the fact that the small molecules in solutions are undergoing Brownian motion and so the distance between the scatterers in the solution is constantly changing with time. This scattered light then undergoes either constructive or destructive interference by the surrounding particles and within this intensity fluctuation, information is contained about the time scale of movement of the

scatterers. Dynamic Light Scattering (DLS) System measures particle size from 1 nanometer to 6 microns at concentrations up to 40% w/v. All DLS systems measure light scattering effects arising from the Brownian motion of particles in suspension. Particle size distribution analyzers based on measuring the phenomenon of Brownian motion can be broadly classified as being based on either autocorrelators or on power spectrums.

Zeta potential

The zeta potential of a system is a measure of charge stability and controls all particle - particle interactions within a suspension. Understanding zeta potential is of critical importance in controlling dispersion and determining the stability of a nanoparticle suspension, i.e. to what degree aggregation will occur over time. The zeta potential is the measure of the electric potential at the slip plane between the bound layer of diluent molecules surrounding the particle, and the bulk solution. This can be closely linked to the particle's surface charge in simple systems but is also heavily dependent on the properties of the diluent solution. A higher level of zeta potential results in greater electro-static repulsion between the particles, minimizing aggregation/flocculation.

TEM analysis

For the confirmation of size and shape transmission electron microscope (TEM) measurements can be carried out using drop coating method in which a drop of solution containing nanoparticles was placed on the carbon coated copper grids and kept under vacuum desiccation for overnight before, loading them onto a specimen holder. TEM is a microscopy technique whereby a beam of electrons is transmitted through a specimen, interacting with the specimen as it passes through. An image is formed from the interaction of the electrons transmitted through the specimen; the image is magnified and focused onto an imaging device, such as a fluorescent screen, on a layer of photographic film, or to be detected by a sensor such as a CCD camera. TEMs are capable of imaging at a significantly higher resolution than light microscopes, owing to the small de Broglie wavelength of electrons. This enables the instrument's user to examine fine detail—even as small as a single column of atoms, which is tens of thousands times smaller than the smallest resolvable object in a light microscope.

SEM analysis

Scanning electron microscopy (SEM) was extremely useful for the determination of topology and observations of surfaces as they offer better resolution and depth of field than optical microscope. SEM is a type of electron microscope that images the sample surface by scanning it with a high-energy beam of electrons in a raster scan pattern. The electrons interact with the atoms that make up the sample producing signals that contain information about the sample's surface topography, composition and other properties such as electrical conductivity. The types of signals produced by an SEM include secondary electrons (SE), back-scattered electrons (BSE), characteristic X-rays, light (cathodoluminescence), specimen current and transmitted electrons.

AFM analysis

The AFM is one of the foremost tools for imaging, measuring, and manipulating matter at the nanoscale. The atomic force microscope (AFM) is ideally suited for characterizing nanoparticles. It offers the capability of 3D visualization and both qualitative and quantitative information on many physical properties including size, morphology, surface texture and roughness. Statistical information, including size, surface area, and volume

distributions, can be determined as well. A wide range of particle sizes can be characterized in the same scan, from 1 nanometer to 8 micrometers.

XRD analysis

X-ray diffraction or crystallography is a method of determining the arrangement of atoms within a crystal, in which a beam of X-rays strikes a crystal and diffracts into many specific directions. From the angles and intensities of these diffracted beams, a crystallographer can produce a three-dimensional picture of the density of electrons within the crystal.

EDS analysis

For Electron dispersive X-ray spectroscopy (EDS), samples prepared on a carbon coated copper grids and kept under vacuum desiccation for three hours before loading them onto a specimen holder. It is used for the determination of elemental composition and purity of the sample by atom %.

XRF analysis

XRF is a technique used for chemical analysis of materials. An X ray source is used to irradiate the specimen and to cause the elements in the specimen to emit (or fluoresce) their characteristic X-rays. A detection system (wave length dispersive) is used to measure the peaks of the emitted X-rays for qual/quant measurements of the elements and their amounts.XRF is routinely used for the simultaneous determination of elemental composition and film thickness.

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CHAPTER 4

FERMENTATION IN FOOD PROCESSING TECHNOLOGY

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ABSTRACT
Fermentation is an important and popular technique in food processing technology. The fermentation technology applicable to food processing sector is also popularly known as zymology or zymurgy. Fermentation is brought about by the conversion of sugars into ethanol chemically (Ganguly, 2012b). It is resulted from the chemical reaction resulting from the breakdown of higher carbohydrates to alcohols and organic acids or alcoholic derivatives.

KEYWORDS: Fermentation, Food processing

INTRODUCTION
Fermentation technology is employed in the bread manufacturing industries for leavening activity brought about by the production of carbon dioxide by the microbial or yeast activity. The preservation effect during fermentation is attributed to the production of lactic acid in sour foods such as yoghurt, dry sausages, pickles, sauerkraut and vinegar (extremely diluted acetic acid) [Steinkraus, 1995; Harden and Young, 1906]. Fermentation is a microbial technique and the reaction to be controlled in favorable and desirable conditions for food safety and quality after fermentation, especially in the production of alcoholic premium quality beverages like beer, wine and cider (Cavalieri *et al*., 2003; Steinkraus, 1995; Ganguly, 2012b).

HISTORICAL PERSPECTIVE
Louis Pasteur, the renowned French chemist is the world famous and first known zymologist in history, who in 1856 established the pivotal role of yeasts in fermentation. Pasteur originally defined fermentation as "respiration without air" after regular performances of lengthy experimental protocols (Dubos, 1995). After observation of the breakdown of sugars to alcohols by the action of yeast, the pioneer concluded that the entire reaction is driven by the chemical catalytic action of certain forces called ferments inside the yeast cells. It was further observed that the yeast extracts can bring bout fermentation of sugars even also in the absence of viable yeast cells. In 1897, Eduard Buchner of Humboldt University of Berlin, Germany discovered that sugars are fermented in the absence of viable cells also in the fermentation mixture. The yeast cells secrete a chemical component called zymase. For his memorable contributions in research and discovery of cell-free fermentation, in 1907 Buchner was awarded with the prestigious Nobel Prize in Chemistry. In 1906, NAD^+ was discovered out of studies carried out from ethanol fermentation.

The fermentation technology under controlled conditions is an age old practice both in households and industries for food processing and preservation, be it alcoholic beverage products of edible products derived from vegetable, fish and meat sources (Steinkraus, 1995).

FERMENTED FISH PRODUCTS AVAILABLE WORLDWIDE

Specifically, in the fish processing technological research aspect, Bagoong, Faseekh, Fish sauce, Garum, Hákarl, Jeotgal, Hentak (Sarojnalini and Vishwanath, 1995), Rakfisk, Shrimp paste, Surströmming,Shidal (Ahmed *et al*., 2013a; 2013b; Muzaddadi and Basu, 2012) and Ngari (Thapa *et al*., 2004) are the popular fermented fish products worth mentioning.

UTILITY OF THE TECHNIQUE

Fermentation technology is primarily employed for the preservation of different food by production of acids and alcohols, biological fortification and enrichment of food items with potential biogenic products like essential amino acids, easily digestible proteins, essential fatty acids and useful vitamins, neutralization of anti-nutritional factors, to diversify and enrich the diet with various aromas, flavours and textures in food substrates and decrease in requirements of further processing techniques like cooking etc (Steinkraus, 1995).

PUBLIC HEALTH RISKS INVOLVED FROM CONSUMPTION

Alaska has witnessed a steady increase of cases of botulism since 1985. It has more cases of botulism than any other state in the United States of America. This is caused by the traditional Eskimo practice of allowing animal products such as whole fish, fish heads, walrus, sea lion, and whale flippers, beaver tails, seal oil, birds, etc., to ferment for an extended period of time before being consumed. The risk is exacerbated when a plastic container is used for this purpose instead of the old-fashioned, traditional method, a grass-lined hole, as the botulinum bacteria thrive in the anaerobic conditions created by the air-tight enclosure in plastic containers (U.S. federal agency Report).

There are certain risks and health hazards associated with excess and regular consumption of fermented food products. In the countries belonging to the Northern hemisphere there has been increase in incidences of botulism exceeding the case reported in the Americas. During the practice of extended fermentation, if plastic wrappers or containers are used, then *Clostridium botulinum* gets a conducive condition to thrive in the micro-aerophilic condition inside the plastic containers (Ganguly, 2012a). This is mainly caused for the practice of allowing whole fish, fish heads and meat of animals like sea lions, walrus, whale flippers, birds, seal tallow, beaver tails etc. to ferment for prolonged periods before consumption by the resident Eskimos there.

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CHAPTER 5

FUNGI: A REVIEW ON MUSHROOMS

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ABSTRACT
This paper reviews a fungus – mushrooms. In this paper, identification, cultivation, uses, side effects, nutritional and medicinal values, storage, marketing and other uses of mushrooms were discussed. From the review too it was observed that its usefulness surpasses the side effects. These side effects could be eliminated if proper 'processing' could be employed. Due to advances in both basic knowledge and practical technology relevant to mushroom farming, mushroom products and mushroom bioremediation, developing countries should harness the potentials of mushrooms as this would boost the revenue income and healthy living. It is hoped that this paper would add to existing information on this fungus.

KEYWORDS: Mushrooms, Nigeria, New employment, Medicinal value

INTRODUCTION

The organisms of the fungal lineage include mushrooms, rusts, smuts, puffballs, truffles, morels, and yeasts, as well as many less well known organisms (Blackwell *et al*, 2011). More than 700, 00 species of fungi have been described however, some estimates of total numbers suggests that 1.5 million species may exist (James *et al*, 2006).

Edible mushroom (Figs 1 & 2) have for a long time been recognized not only as a delicacy, but also for their use as food in man's diets. Mushrooms have been found to be rich sources of protein, lipids, amino acids, glycogen, vitamins and mineral elements (Okhuoya *et al*., 2010). According to Rambeli (1983), the mineral salt content of mushrooms is superior to that of meat and fish and nearly twice that of the most common vegetables.

Nigeria is a country of many tribes, the Hausa in the North, Yoruba in the West, Urhobo in the Mid – West and the Ibo in the East, to mention a few. Each tribe has recognized mushrooms for many years, and the people have made use of a number of them economically in their daily life.

The Yorubas have recognized mushrooms for many years as in fungi have always played an important role in their everyday life. They have descriptive Yoruba names for their species of mushrooms as well as mythical stories and beliefs, which explain the origin of some of them. These myths and beliefs sometimes play a role in determining which of the mushrooms are edible and which of them may be used for medical purposes by the Yoruba native doctors.

The nutritional and medicinal values of mushrooms have long been recognized. In recent times, however, mushrooms have assumed greater importance in the diets of both rural urban dwellers. For example, they are being marketed along major highways and urban centers where the trade now booms. It is conceivable that the increased demand for

mushrooms is contingent upon the phenomenal rise in the unit costs of the conventional sources of meat (e.g beef, pork, chicken, etc).

Edible mushrooms have been placed into five categories according to the derivation of their names, viz., those named according to the taste, morphology, growth habit, texture, and habitat (Oso, 1975). Examples in each category are: taste (*Volvariella volvacea, Volvariella esculenta* Yor. Ogiri agbe) *Termitomyces clypeatus* (Yor. Takete): Morphology (*Termitomyces manniformis*) (Yor. Rooro) *Termitomyces robustus* (Yor.Ewe) *Schizophyllum commue* Fr (Yor. Ese-adie) *Agrocyber broadway* (Murr) (Yor. Gunnugu); growth habit (*Termitomyces globulus, Pleurotus tuber-reguim*) (Yor. Olu); texture (*Pleurotus squarrouslus*) (Yor. Erirokiro), *Psathyrella atroumbonata* (Yor.Wowo); habitat (*Francolimus bicalcaratus*) (Yor.Isoaparo). In addition to the above, the natives have observed the growth of many fungi on different kinds of dead wood and have named each fungus after the wood on which it grows.

Besides the edible mushrooms, the natives also recognize some poisonous or none edible fungi a few of which are listed here. *Coprinus africans* (Yor. Ajeimutin), *Phallus aurantiacus, Phallus industiatus, Phallus rubicundus* and *Mutinus bambustnus* (Yor.Akufodewa), *Celtis zenkeri* (Yor.Asa-ita), *Coprinus enphemerus* (Yor.Olu-gbongaga).

There has been a recent upsurge of interest in mushrooms not only as a health vegetable (food) which is rich in protein but also as a source of biologically active compounds of medicinal value. Uses include complementary medicine/dietary supplements for anticancer, antiviral, immunopotentiating, hypocholesterolemic, and hepatoprotective agents. This new class of compounds, termed *mushroom nutriceuticals* are extractable from either the mushroom mycelium or fruiting body and represent an important component of the expanding mushroom biotechnology industry. It has been shown that constant intake of either mushrooms or mushroom nutriceuticals (dietary supplements) can make people fitter and healthier. In addition, mushroom cultivation can also help to convert agricultural and forest wastes into useful matter and reduce pollution in the environment. Therefore, mushroom cultivation can make three important contributions: production of health food, manufacture of nutriceuticals, and reduction of environmental pollution.

Mushrooms are among the largest fungi, which have attracted the attention of naturalists before microscopes, or even simple lenses had thought of. Mushrooms date back to antiquity and are even associated with some past. For example, the Romans attributed the appearance of mushrooms and truffles to lightning hurled by Jupiter to the earth. Even, the Indians of Mexico and Guatemala believe that the appearance of certain mushrooms such as the “fly agaric” *Amanita muscaria* is correlated with a relationship between thunder, lightning and the earth.

CEREMONIAL USES OF MUSHROOMS

For centuries, some mushrooms have been used in religious ceremonies of many ancient people and primitive tribes.

Mushrooms are believed by the Romans to have properties that could produce super human strength, help in finding lost objects and lead the soul to the realm of the gods (Grube *et al.,* 2001). Here in Nigeria, the people of Ohia, in Abia State during one of their festivals consume the fungi (Lebo, 2004).

CULTIVATION OF MUSHROOMS

As a group, mushrooms occur in different parts of the world, ranging from arctic to the tropics. While some species occur only in restricted areas, others exist in areas that are widely separated geographically. However, most species seem to show a preference for a certain type of habitat. Some are, for instance, found primarily in upland wooded areas; others exist in swamps and still others prefer open areas such as gardens, lawns or pasture e.g *Pleurotus tuber-regium*. Many species particularly the mycorrhizal forms are associated with certain types of vegetations. Within a certain habitat, mushrooms may also show a preference for a particular substratum. Basidiocarps of some are typically produced on the soil and are generally referred to as terrestrial forms. Others are found on dead leaves (follicolous) or litter e.g *Cortinarius melliolens* and *Tricholoma lobayensis*, on wood (lignicolous) e.g *Lentinus edodes* or on dung (corpophilous) e.g *Coprinus lagopus*. A few grow on nasidiocarps of other mushrooms and are termed fungicolus.

Mushrooms are mostly found on wastes such as sawdust, garbages and composting materials (Gbolagade *et al*., 2006). Statemet (2001) noted that garden mushrooms were propagated from fermented horse dung and moist litter all the year round. Miles and Chang (2004) deduced from the evidence presented in various early publications, that mushroom cultivation started in France about 1630, since then, series of studies have been conducted to monitor different growth parameters in both natural and pure culture. Compositing progress was measured by changes in temperature, hydrogen ion concentration (pH), and ability of compost top support growth of mushroom mycelia.Growth of mushroom was observed to take place over a wide range of pH, i.e pH 3.4 – pH 9.0 (Adebayo *et al*., 2009). The investigation carried out on *Coprinus cinerus a*nd *Volvariella volvacea* in relationship to pH showed that a drop in pH enhanced the growth of *C. cinerus* while neutral pH favors *V. volvacea*.

A combination of condition such as temperature, pH, light, physical properties of subtraction and proportion of nutritional factors have effect on the growth of mycelia as well as production of fruiting bodies.

During mycelia growth on their substrate, mushroom produce a number of enzymes which breakdown complex organic compounds such as cellulose and lignin into soluble production, which are absorbed by the hyphae. Different types of mushrooms were qualitatively examined for their ability to hydrolyse starch. Kuforiji and Fasidi, (2008) proved that *Lentnus adodes* mycelia are known to grow on various agar media and liquid cultures. Composts supplemented with organic material containing vitamin B-complex were claimed to enhance greater yield of mushroom.

The general pattern carbon source utilization of *Agaricus bisporus* is similar among the various reports despite considerable difference in strains, time and place of study. Generally glucose, fructose and xylose are reported as good carbon sources. All fungi require a nitrogen source.

Investigations on the effect of several environmental factors on mushroom both independently and in different conditions were carried out by Adenipekun and Fasidi, (2005); Kuforiji and Fasidi, (2007); Ukoima *et al*., (2009a & b). They found out that a combination of condition such as temperatures, pH, physical properties of substratum of proportion of nutritional factors have effect on the growth of mycelia. Mycelia are produced

as a result of spore germination. It is the fruit body that attracts the attention of mushroom hunters because it is fleshy and also more conspicuous.

Lots of researches have been carried out in Nigeria. Some of the works include cultivation on sawdust of different plants (Okhuoya (1998); Gyar and Ogbonna (2006); Kuforiji and Fasidi, (2008), agricultural and agro industrial wastes (Fasidi and Kadiri, (1993); Adenipekun and Fasidi (2005); Ayodele and Okhuoya (2007); Kuforiji and Fasidi, (2007); Onuoha *et al*. (2009); Ukoima *et al*. (2009), cotton waste and cassava peel (Adebayo *et al*. 2009) and culture media (Ukoima *et al*. 2009). Elsewhere, examples of the different formulas for spawn substrates are: Mother grain spawn: (i) Wheat/rye grain C 1.5% gypsum or slaked lime. (ii) Cotton seed hull 40%, sawdust 38%, wheat bran 20%, sugar 1%, and gypsum 1%. (iii) Sugar cane bagasse 40%, sawdust 38%, wheat bran 20%, sugar 1%, and gypsum 1%. Planting spawn: A number of materials, mostly agricultural and forest wastes, can be used to prepare mushroom planting spawn. Three of them are given here as examples: sawdust 78%, rice/wheat bran 16%, sugar 1.5%, corn flour 1.7%, ammonium sulphate 0.3%, calcium superphosphate 0.5%, and gypsum 2%; sawdust 64%, wheat bran 15%, spent coffee grounds 20%, and gypsum/lime 1%; and sawdust 78%, sucrose 1%, wheat bran 20%, and calcium carbonate 1%.(Chang, 2008).

Apart from cultivation from wastes, there is the mushroom industry. Mushroom industry can be considered to be composed of cultivated edible mushrooms, medicinal mushrooms, and wild mushrooms (Chang, 2006).

According to the Training manual on Mushroom Cultivation Technology, (2008), there are advantages of mushrooms cultivation, these are highlighted below:

1. Wastes such as cereal straws are largely burnt by the farmers, which causes air pollution. However, these raw materials can actually be used for the cultivation of mushrooms. This kind of bioconversion exercise can greatly reduce environmental pollution.
2. It serves as means of generating employment, particularly for rural women and youths in order to raise their social status.
3. It provides the people with an additional vegetable of high quality, and enrich the diet with high quality proteins, minerals and vitamins which can be of direct benefit to the human health and fitness. The extractable bioactive compounds from medicinal mushrooms would enhance human's immune systems and improve their quality of life.
4. Mushroom cultivation is a cash crop. It improves economic standards of the people some warm mushrooms, e.g. *Volvariella volvacea* (Straw mushrooms) and *Pleurotussajor-caju* (Oyster mushrooms) are relatively fast growing organisms and can be harvested in 3 to 4 weeks after spawning. It is a short return agricultural business and can be of immediate benefit to the community.

IDENTIFICATION

Macroscopic structure of mushrooms must be understood before identification can be made. Most of them are Basidiomycetes and gilled. Their spores called basidiospores are produced on the gills and fall in a fine rain of powder from under the caps as a result. At the microscopic level the basidiospore are short off basisia and then falls between the gills in the dead air space. As a result, for most mushrooms, if the cap is cut off and placed gill – side – down overnight, a powdery impression reflecting the shape of the gills is formed. The color

of the powdery print, called a spore print is used to help classify mudrooms and can help to identify them. Spores prints colors include white, brown, black, purple-brown, pink, yellow and cream, but almost never blue, green, or red.

A good reference material, usually a book with color, pictures of the different mushrooms known, is a basic requirement. A key is usually provided to simplify identification in most reference texts (Carlucccio, (2003); Fuhrer, (2005). In using the reference, it is essential that one knows some specific characteristics of the mushroom being identified. These characteristics are (1) size, color, and consistency of the cap and the stalk; (2) mode of attachment of the gills to the stalk; (3) spore color in mass; and (4) chemical tests or reactions.

There is no single reference work in which all mushrooms are illustrated or described. In most cases, mushroom species in publications are grouped by region or locality, for example, North American mushrooms, mushrooms of the Western Hemisphere, mushrooms of South Africa and those found in Nigeria. While certain mushrooms are easy to identify, many are not. In fact, there are a great number of look-alikes. To avoid any unpleasant experiences, especially when identifying mushrooms for the purpose of determining edibility, experts should always be consulted.

NUTRITIONAL VALUES OF MUSHROOMS

Edible mushrooms are important sources of food. They form very nourishing meals especially for invalids, for they are easily digestible. They are consumed not only for their innate flavour and taste, but also for their important nutritional value. On fresh weight basis mushrooms are superior in protein content (Aremu *et al*., 2009) to all vegetables and fruits, but are inferior to meat and dairy products, which are the conventional protein sources. On dry-weight basis, however, mushrooms are similar with respect to dried-yeast and superior to dried peas and beans. The nutrient content varies from species and depends on their growth requirement. Mushrooms have a high percentage of water 93-95% as compared to learn beef (70%) and fresh vegetables (92%). They also contain valuable minerals such as iron, potassium, phosphorus, calcium and copper, 56% carbohydrate, 30% protein, 2% fat and also 10% ash on dry weight basis. They are also rich in vitamin B and vitamin D.

Mushrooms provide a high protein and low caloric diet and can thus be recommended to heart patients. They also contain all the essential amino-acid required by an adult (Koyyalamudi *et al*., 2009). Tryptophan and lysine are present in high concentrations as compared to cystein and methionine.

Mushrooms is reported to be an excellent source of riboflavin and nicotinic acid; a good source of pantothenic acid and ascorbic acid (Ukpebor *et al*., 2007). The carbohydrate and fat contents of edible mushrooms are quite low. The absence of starch in mushrooms makes it an ideal food for diabetic patients and for persons who wants to shed excess fat.

Edible mushrooms known as the meat of the vegetable world (Haas and James, 2009) can be prepared into a variety of delicious dishes and as flavours for other dishes. Among the Nigerian mushroom dishes are mushrooms with vegetable, mushroom with vegetable and melon soup, mushroom in okro soup, and mushroom in stew. These soups are used to eat a variety of foods. Some people use mushrooms as a substitute for meat in their stews (Abulude, 2005).

There is evidence that consumption of plant foods such as fruits and vegetables, provide protection against various diseases, especially chronic degenerative diseases (Selvi *et al* (2007). This protection can be explained by the free –radical scavenging capacity of antioxidants in plant foods. Plant foods are a good source of polyphenols, which have been reported to be effective radical scavenger and inhibitors of liquid peroxidation (Makam and Konig, 2001). Kettawan *et al*., (2011) and Selvi *et al* (2007) have demonstrated that mushrooms contain antioxidants.

Apart from their nutritive values, mushrooms also have potential medicinal benefits especially as antitumour. Abulude, (2005); Kuforiji and Fasidi, (2008) and Kettawan *et al*., (2011) elaborated on the medicinal uses of Pleurotus tuber-regium in Nigeria. The stated that these mushrooms can be used in combination with other herbs as ingredients to care ailments such as chest pain, cold, dropsy, fever, headache, smallpox and stomach pains. The low carbohydrate content of mushrooms makes it an ideal food for diabetics and people w1ho intend to control their body weight.

SIDE EFFECTS OF MUSHROOMS

Mushrooms can also cause disease, decay and destruction in their search for food, since they have no chlorophyll, they must obtain organic material in prepared form, for example, *Armillaria mellea* "Honey fungus" causes serious damage to ramiferous tress and may attack shrubs and even herbaceous plants such as potatoes, and strawberries (Lange and Hora, 1963).

Cultivation of mushrooms on commercial scale is new to many developing countries of which Nigeria is one. This fact is due to the scantiness of information on the growth requirement of many indigenous mushrooms. In Nigeria, there are lots of industrial and agricultural wastes that may serve as potential source of raw material for commercial cultivation of mushroom at minimum cost.

Ita *et al,*(2008) in their study on bioaccumulation potential of heavy metals in sporocarps from Niger Delta region of Nigeria, revealed that certain mushrooms accumulate heavy metals. The accumulating potentials are affected by the species, substrate composition, age of mycelium and intervals between fructifications. Studies on metals in mushrooms have shown a correlation between fungal metals concentration and point sources of metal pollution (Isildak *et al*, 2004; Gyar and Ogbonna, 2006).

Human activities have been reported to impact negativity on arable lands contaminating them with pesticides, petroleum hydrocarbons, heavy metals and waste engine oil pollutants, and consequently causing arable land shortage and other environmental challenges (Okhuoya *et al*, 2010; Oghenekaro *et al*, 2008). These challenges may exert negative effect such as kidney damage, impairment of circulatory, reproductive and nervous system damage (Abulude *et al*, 2004) on man and animals when consumed (Ita *et al*, 2008).

Apart from the side effects, Mushroom mycelia can produce a group of complex extracellular enzymes which can degrade and utilize the lignocellulosic wastes in order to reduce pollution. It has been revealed recently that mushroom mycelia can play a significant role in the restoration of damaged environments. Saprotrophic, endophytic, mycorrhizal, and even parasitic fungi/mushrooms can be used in mycorestoration, which can be performed in four different ways: mycofiltration (using mycelia to filter water),

mycoforestry (using mycelia to restore forests), mycoremediation (using mycelia to eliminate toxic waste), and mycopesticides (using mycelia to control insect pests). These methods represent the potential to create a clean ecosystem, where no damage will be left after fungal implementation (Training Manual on Mushrooms cultivation, 2008).

STORAGE OF MUSHROOMS

At the post-harvest stage, mushrooms need to be stored in fresh condition to maintain their qualities and flavor, some common preservation methods are enumerated below which increase the shelf life of harvested mushrooms. Storage in 0.02-0.03mm dense polythene bags with nitrogen at 0^0C is equivalent to 5 weeks of shelf life; storage at 50^0C will give the mushroom a shelf life of 4 weeks while storage at 15^0C under the same condition will preserve the fungi for 2 weeks. It is noted that storage in controlled atmosphere of 9% oxygen and 25% carbon-dioxide; preservation by gamma-radiation will preserve mushroom for 10 days. Mushrooms can also be freeze-dried. Dehydration, grinding and storage in an air tight container could also be employed. The stored powder can be used for making mushroom soup, mushrooms can be treated and canned (Roy, 1984).

MARKETING

About 160,000 metric tons of mushrooms are produced annually in Japan, half of which is dried and exported. It represents a two billion dollars industry which employs about 200,000 people (Anderson and Marconiller, 2006). Worldwide, the business is worth millions of pounds annually, for the countries of Eastern Europe in particular, wild mushrooms are precious exports. Poland and France are two of the major exporters of mushrooms. In a statement from Allbusiness.com (2003), China is the world's largest edible mushroom producer. The country produces about half of all cultivated mushrooms, and around 2.7 kilograms of mushrooms are consumed per person per year by over a billion people.

Authoritative source from Spore (2006) revealed economically, mushrooms growers' association in Uganda sold 44 tons per year to Japan, 40 tons to the US and 2 tons to the Democratic Republic of Congo.

According to Olubanjo *et al*, 2006 in Nigeria, the commercial production and trade s still in its infancy. They attributed this to poor underdeveloped nature of demand for edible, local and cultivated exotic mushrooms. To increase the mushroom marketing, constant advertisement to create awareness, good storage and packaging should be ensured. Good storage and value addition by processing and canning can solve the problem of seasonality in availability (Olubanjo *et al*, 2006). Gradually, mushrooms are income generating.

MEDICINAL VALUE

Medicinal mushrooms are mushrooms, or mushroom extracts, that are used or studied as possible treatments for diseases. *Lentinula edodes* (shiitake), *Grifola frondosa* (maitake), and *Ganoderma lucidum* (reishi), have a history of medicinal use spanning millennia in parts of Asia. Medicinal mushroom research has indicated possible cardiovascular, anticancer, antiviral, antibacterial, antiparasitic, anti-inflammatory, hepatoprotective, and antidiabetic activities (Lentinan, 2009).

In Nigeria benefits of mushrooms such as the nutrition, medicinal and mythological uses have been reviewed (Akpaja *et al*., 2005; Osemwegie *et al*., 2006). Even Labarere and Menini (2000) acknowledged that the uses of mushroom genetic resources are not only of high

interest in agronomy, agriculture, human food and animal feed but also for the discovery, production and development of molecules or components with high added value in industries such as chemical and pharmaceutical industries.

Some mushrooms materials, including polysaccharide, glycoprotein and proteoglycens, modulate immune system responses and inhibit tumor growth. Currently, several extracts have wide spread use in Japan, Korea, and China, as adjuncts to radiation treatments and chemotherapy (Smith *et al*, 2002; Borchers *et al*, 2008). Mushrooms that have psychoactive substances have been used as sacraments for healing (Mental and physical) (Hudler 2000). Certain mushrooms, especially polypores like Reishi were thought to be able to benefit a wide variety of health ailments (Sarfaraz *et al*, 2009).

Ogbe *et al*., (2008), in a research carried out, they found out there were improvements of egg-laying and disease resistant capacity of birds when they used *Ganoderma* species. Beta-glucan based dietary supplements of mushroom origin are effective for the treatment of Buruli ulcer caused by *Mycobacterium ulcerans* in Ghana while *Ganoderma lucidum* (Leyss.) Karst. Tested in separate study for the treatment of *Eimeria tenella* infected broiler chickens in Nigeria (Okhuoya *et al*., 2010).

OTHER USES

According to Wikipedia, the free encyclopedia (2011), mushrooms have been used for dyeing wood and other natural fibers. The chromosphores of mushroom dyes are organic compound and produce strong and vivid colors, and all colors of the spectrum can be achieved with mushrooms dyes. Dyes from them have been the source of many dyes before the synthetic ones (Mussak and Bechtold 2009).

In the US, and other developed countries, mushrooms have been used as fire starters. They have also been applied by Ecovative design LLC to produce biodegradable packaging. Presently, they play a role in the development of new biological remediation techniques and filtration technologies (Wikipedia, 2011)

Mushrooms are used as gun powder (Akpaya *et al*., 2005)

  

Agaricus arvensis *Chloropyllum* sp. *Macrolepiota* sp.

  

Pycnoporus cinnabarinus *Tremella fuciformis* *Pleurotus porrigens*

  

Pleurotus squarrosolus *icholoma* sp *Daldiniacon centrica*

  

Russula sp. *Schizophyllum commune* *Scutellinia sp*

Fig 1 : Some mushroom resources found in Nigeria (Source: Osemwegie,O.O, 2006)

Thelephora sp   *Geastrum saccatus*   *Microstoma* sp.

Unknown   *Auricularia auricula*.

Fig 2 : Some mushroom resources found in Nigeria (Source: Osemwegie,O.O, 2006)

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CHAPTER 6

PROPER NUTRITION AND FEED ADDITIVES FOR IMMUNOMODULATION FOR SUSTAINABLE AQUACULTURE AND IN REDUCTION OF HARVEST AND POST HARVEST LOSSES

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INTRODUCTION

For preparing and formulating fish feed the economic aspect of each and every ingredient needs to get proper priority, as it is well known that appreciable amount of nutritional loss occurs during processing, heat treatment and storage. The nutritional requirements for fish mainly depends on the rate of growth of the fish in conjunct with additional influences like size, metabolic function in addition to the environmental influences and management strategies employed for rearing and breeding (Ganguly, 2013b).

Ideal aquafeed should contain adequate nutritional ingredients should be formulated as to contain all the essential components which should be balanced and adequate for the proper maintenance of growth, reproduction and overall health of the fishes (Ganguly, 2013b).

Diet for the fishes should be devoid of the harmful antinutritional factors (including mycotoxins majorly, aflatoxicosis, ochratoxicosis and zearalenone) which deteriorate the quality of the diet. The formulated diet should be well acceptable to the fishes and should not pose any adverse effect on their habitat or water system (Gatlin et al., 1986).

A variety of polysaccharides from many sources behave as immunomodulators by stimulating the immune system. Increases in the interest in glucans as a result of experimental evidences have shown that 'zymosan' has the ability to stimulate macrophages by activating the complement system. They can be pharmacologically classified as biological response modifiers (BRM). Biological activities of β-1,3-glucans is influenced by different physicochemical parameters, such as solubility, primary structure, molecular weight, branching and polymer charge. During the development of immune reactions, immunomodulating effects of β-glucans are well established (Vetvicka and Sima, 2004). β-1,3-glucan possessa strong immunostimulating activity in a wide variety of species, including shrimps, fish (Ganguly et al., 2009, 2010a), rats ,rabbits, guinea pigs, sheep, pigs , cattle, humans. Based on these results it has been concluded that β-1,3-glucans represent a type of immunostimulant that is active across the evolutionary spectrum. Invertebrates have active defense mechanisms which enable them to use their highly effective innate defense pathways against invading pathogens despite the absence of lymphocytes or antibody based adaptive immune system (Vetvika et al., 2004; Ganguly et al., 2009, 2010a, 2010b, 2013a).

Requirement at different stages of growth

The nutrients in the aquafeed should be highly digestible to the fishes with high bioavailaibility. The feed should also have high storage life and losses due to physical and climatic factors should be minimum (Ganguly, 2013b).

The nutritional requirement among various fish types vary as per their habitat from freshwater to brackish water and to the marine system (Garling and Wilson, 1976). Fishes have varying nutritional requirement depending on their growth phase i.e. from larval, fishling, spawning and up to table fish stage. During the period of maximum growth, the requirement for potential nutrients also rises (Cowey, 1975).

Role of optimum availability of proximate principles

The availability of minerals varies among various fish species and sources. Phosphorus digestibility in some feeds by channel catfish or rainbow trout is much higher than by the stomachless carp.

The fatty acid content also varies linearly with it. The vitamin and mineral composition should be in conjunct with the major proximate principles present in the aquafeed. The energy presence in the diet varies according to the size of the fish species as it is met up by the presence of carbohydrate content (Cho and Kaushik, 1985).

Under normal processing and storage conditions the amino acids, several vitamins and inorganic nutrients are relatively stable to heat, moisture and oxidation. Some of the vitamins are recommended for use in excess of the requirement, as they are subject to some loss. On the contrary, excess fortification with vitamins and micronutrients beyond permissible limits may lead to some loss in the nutrient content majorly during feed processing (Ganguly, 2013b).

The protein incorporation in the formulated diets should be in optimum ratio with the energy component present therein. Majorly, the protein component should consist of all the essential amino acids (Cho and Kaushik, 1985). The technical or reagent grade compounds fetch more mineral sources and are more consistent than from usual feedstuffs (Goldstein and Forster, 1970). In the diet formulation for fishes, the protein finds the upper hand as the most important proximate component in the diet. The overall digestion coefficient of the diet depends on the availability of superior and qualitative protein source.

Feed additives for immunomodulation

Biological activities of β-1,3-glucans is influenced by different physicochemical parameters, such as solubility, primary structure, molecular weight, branching and polymer charge. During the development of immune reactions, immunomodulating effects of β-glucans are well established (Vetvicka and Sima, 2004). β-1,3-glucan possessa strong immunostimulating activity in a wide variety of species, including shrimps,fish ,rats ,rabbits, guinea pigs, sheep, pigs , cattle, humans. Based on these results it has been concluded that β-1,3-glucans represent a type of immunostimulant that is active across the evolutionary spectrum. A variety of polysaccharides from many sources behave as immunomodulators by stimulating the immune system. Increases in the interest in glucans as a result of experimental evidences have shown that 'zymosan' has the ability to stimulate macrophages by activating the complement system. They can be pharmacologically classified as biological response modifiers (BRM). Invertebrates have active defense mechanisms which enable them to use their highly effective innate defense pathways against invading pathogens despite the absence of lymphocytes or antibody based adaptive immune system (Vetvika et al.,2004; Ganguly et al., 2009, 2010a, 2010b, 2013a).

β-1,3-glucans are usually isolated from cell walls of bacteria, mushrooms, algae, cereal grains, yeasts and fungi and are structurally complex homopolymers of glucose (Zekovic and Kwiatowski, 2005).

Anti-microbial immune mechanisms found in invertebrates can be induced by fungal β-glucans (Brown and Gordon, 2005). Protease cascades initiated by PAMP induce majority of recognition of these responses in the haemolymph. Cascade activation results in coagulation in the haemolymph, or anti-microbial peptide secretion by immunocompetent cells. β-glucan recognition can also result to phagocytosis by certain haemocytes . β-glucans are recognized by fishes as foreign agents because of their similarity to fungal or bacterial Gram negative polysaccharides. An inflammatory response is produced as a result by the immune system of fishes after exposure to provide effective protection. (Robertsen et al., 1994).

Innate immunity plays a very important role in combating microbial infection in all animals. Surface determinants conserved among microbes are recognized by receptors which activate the innate immune response, but absent in lipopolysaccharides, peptidoglycans and mannans (Medzhitov and Janeway, 1997, Ganguly et al., 2009, Ganguly et al., 2010b). These receptors upon recognition activate multiple and complex signalling cascades leading to regulation of transcription of target genes encoding effector molecules. Specific transcription programmes elicited by different pathogens can be investigated by using microarray technology (De Gregorio et al., 2001). Diseases possess a major problem in aquaculture production, especially for the invertebrate farming (Ganguly et al., 2009, 2010a, 2010b, 2013a).

Under intensive conditions, fishes are more susceptible to microbial infections especially in their larval stages. During stress, immunostimulants can provide resistance to pathogens. Only a few immunostimulants can be used in aquaculture. Glucans are commercially significant as immunostimulating agents. Different types of β-glucans have been used successfully to increase resistance of fish and crustaceans against bacterial and viral infections (Cook et al., 2003; Bagni et al., 2005). It has been seen that health, growth and general performance of many different animal groups, including farmed shrimp, fish and land animals may be improved by the use of β-glucans. Product source, animal species, development stage of the target organism, dose and type of glucan, route and time schedule of administration affect the immunomodulatory effects of glucans (Guselle et al., 2007; Ganguly et al., 2009, 2010a, 2010b, 2013a) and the association with other immunostimulants. Many studies have been carried out to measure the effects of glucan on fish immunity. Some investigators have adopted the *in vitro* culture of macrophages with glucan (Cook et al., 2003), but *in vivo* studies have been carried out by majority of the workers (Sahoo and Mukherjee, 2001).

CONCLUSION

β-glucans can be used as environmental friendly agents, in contrast to the chemical anti-microbial products in aquaculture which is frequently affected with disease problems under intensive conditions. Nowadays, the demand for health promoters, higher feed efficiency and alternatives to antibiotics has increased in aquaculture. β-glucans possess probiotic effects and have immunomodulating activities. Many studies are been done which focus on the properties of these glucans and their efficient use in fish farming (Ganguly et al., 2009, 2010a, 2010b, 2013a).

Preparation of proper and optimum dietary formulation for fishes is a primary prerequisite for sustainable and profitable aquaculture, especially in consideration of the long term economic benefit for the rural fisherfolk community (Ganguly, 2013b).

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CHAPTER 7

GENOMIC DNA EXTRACTION METHOD FROM *Annona senegalensis* Pers. (Annonaceae) FRUITS

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ABSTRACT
Extraction of DNA in many plants is difficult because of the presence of metabolites that interfere with DNA isolation procedures and downstream applications such as DNA restriction, replications, amplification, as well as cloning. Modified procedure based on the hexadecyltrimethylammoniumbromide (CTAB) method to isolate DNA from tissues containing high levels of polysaccharides. The procedure is applicable to both ripped and unripe fruits of Annona senegalensis. This modified CTAB(2%) protocol include the use of 1.4 M NaCl, 1% polyvinylpyrrolidine (PVP), 1% mercaptoethanol and 100% absolute ethanol in the extraction as well as reducing the centrifugation times during the separation and precipitation of the DNA. This method solved the problems of DNA contamination, degradation and low yield due to binding or co precipitation with starches. The isolated DNA proved amenable to PCR amplification and restriction digestion. This technique is fast, reproducible, and can be applied for SSR-PCR markers identification.

KEY WORDS: Annona senegalensis, Genomic DNA, Fruits, Modified, Markers.

INTRODUCTION

*Annona senegalensis***Pers.** is a member of the Annonaceae family and it is a species of seed vegetable which grow both on dry and raining seasons. It is a savannah plant which is widely spread from Senegal to Nigeria, also in Central African Republic (Abdullahi *et al.*, 2003). It produces seeds which are ovate in shape, very small in size and open by mechanical explosion.

A. senegalensis is common in Southern part and in Niger State of Nigeria; where they use the seeds and fruits in making soup (soup harder). The stem, bark, leaves, fruits and roost of *A. senegalensis* have medicinal properties, it may be use in the treatment of cancer, cough and for wound dressing (Abdullahi *et al.*, 2003).The neglect of some local vegetables coupled with the growing reduction in their consumption prompted this research.

The application of DNA technology in agricultural research has progressed rapidly over the last twenty years, especially in the area of cultivar identification (Nybom, 1990). Isolation of plant nucleic acids for use in Southern blot analysis, polymerase chain reaction (PCR) amplifications, restriction fragment length polymorphisms (RFLPs), arbitrary primed DNA amplifications (RAPD, SSR-PCR), and genomic library construction is one of the most important and time-consuming steps. The degree of purity and quantity varies between applications.

A good extraction procedure for the isolation of DNA should yield adequate and intact DNA of reasonable purity. The procedure should also be quick, simple and cheap. The extraction process involves, first of all, breaking or digesting away cell walls in order to

release the cellular constituents. This is followed by disruption of the cell membranes to release the DNA into the extraction buffer. This is normally achieved by using detergents such as sodium dodecyl sulphate (SDS) or cetyl-methyl ammonium bromide (CTAB). The released DNA should be protected from endogenous nuclease. EDTA is often included in the extraction buffer to chelate magnesium ions, a necessary co-factor for nucleases, for this purpose.

The initial DNA extracts often contain a large amount of RNA, proteins, polysaccharides, tannins and pigments which may interfere with the extracted DNA and difficult to separate (Puchooa, 2004). Most proteins are removed by denaturation and precipitation from the extract using chloroform and/or phenol. RNAs on the other hand are normally removed by treatment of the extract with heat-treated RNase A. Polysaccharide-like contaminants are, however more difficult to remove.

They can inhibit the activity of certain DNA-modifying enzymes and may also interfere in the quantification of nucleic acids by spectrophotometer methods (Wilkie *et al.,* 1993). NaCl at concentrations of more than 0.5 M, together with CTAB is known to remove polysaccharides (Murray and Thompson, 1980; Paterson *et al.,* 1993).The concentration ranges mentioned in literature varies between 0.7 M (Clark, 1997) and 6 M (Aljanabi *et al.,* 1999) and is dependent on the plant species underinvestigation. Some protocols replace NaCl by KCl (Thompson and Henry, 1995).

The problem of DNA extraction is still an important issue in the field of plant molecular biology. Various plants contain high levels of polysaccharides and manytypes of secondary metabolites affecting DNA purification. Antioxidants are commonly used to deal with problems related to phenolics. Examples include mercaptoethanol, Bovine Serum Albumin, sodium azide and PVP amongst others (Dawson and Magee, 1995; Clark, 1997). Phenol extractions when coupled with SDSare also helpful. However, with plants having a high content of polyphenolics, SDS-phenol tends to produce low yields of DNA (Rezaian and Krake, 1987).

Several Laboratories involved in the project performed side-by side comparison of all four DNA isolation procedures. Two methods are based on classical principles of lyses and purification. The first one is the commonly used protocol of Doyle and Doyle (1990), which has been used successful in many plant species. The second one, from Guillemot and Maréchal-Drouard (1992) originated from Dellaporta *et al*. (1983) and was modified according to Ziegenhagen *et al.* (1993).

Since the mid 1980s, genome identification and selection has progressed rapidly with the help of PCR technology. A large number of marker protocols that are rapid and require only small quantities of DNA have been developed. Three widely-used PCR-based markers are RAPDs (Williams *et al.,* 1990), SSRs or micro satellites (Tautz, 1989), and AFLPs (Vos *et al.,* 1995). Each marker technique has its own advantages and disadvantages. The choice of a molecular marker technique depends on its reproducibility and simplicity. The best markers for genome mapping, marker assisted selection, phylogenic studies, and crop conservation has Zidani *et al.* (1863) low cost and labour requirements and high reliability. Since 1994, a new molecular marker technique called inter simple sequence repeat (ISSR) has been available (Zietkiewicz *et al*., 1994). ISSRs are semi arbitrary markers amplified by PCR in the presence of one primer complementary to a target micro satellite.

Therefore, the aim of this work is to determine the genomic DNA extraction method from *Annona senegalensis* fruits; thus providing a protocol for purification of high DNA quality and increase productivity of the plant biologically.

Abbreviations: CTAB; hexadecyltrimethylammonium bromide, ISSR, inter-simple sequence repeat, PCR; polymerase chain reaction, SSRs; simple sequence repeats, TBE; tris-borate-EDTA; PVP, polyvinylpyrrolidine

MATERIALS AND METHOD

Several experiments were carried out, however, only the optimised
Protocol is described here.

Plant material

Both ripped and unripe fruits of *A. senegalensis* Pers. were collected from a forest in Kachia Kaduna State Nigeria.

Solutions

An extraction buffer consisting of 2% CTAB (w/v), 100 mM Tris (pH 8.0), 50 mM EDTA (pH 8.0), 1.4 M NaCl, 1% polyvinylpyrrolidine (PVP), 1% mercaptoethanol (v/v), and 3 M sodium acetate (pH 5.2), was prepared. In addition, chloroform: isoamylalcohol (24:1), 75% and 100% ethanol**3** and a TE buffer consisting of 10 mM Tris (pH 8.0) and 1 mM EDTA (pH 8.0) were also prepared.

DNA isolation protocol

Fruits were harvested and frozen immediately in liquid nitrogen. The use of lyophilized tissues offers several advantages.
Dry tissue can be efficiently disrupted while the DNA is unhydrated and can be stored for several years with little loss of DNA quality. A 0.3 g of fruit sample was ground in liquid nitrogen using a mortar and pestle.

The pulverized fruits were quickly transferred to liquid nitrogen.2 % of CTAB buffer (1 ml) containing 1% (v/v) mercaptoethanol and 1% PVP was quickly added to the micro centrifuge tube (2 ml) and stirred with a glass to mix. The tube was incubated at 60 °C for 30 min with frequent swirling. An equal volume of chloroform, Isoamylalcohol (24:1) was added and centrifuged at 10 000 rpm and 4 °C for 15 min to separate the phases. The supernatant was carefully decanted and transferred to a new tube. The above steps, beginning with the addition of chloroform: isoamylalcohol (24:1) and ending with decanting of supernatant, were repeated twice. The supernatant was precipitated with 2/3 volume of ethanol. The precipitated nucleic acids were collected and washed twice with the buffer (75% ethanol, 3 M sodium acetate, TE) (The tubes should not be shaken vigorously because DNA is very vulnerable to fragmentation at this step).

The pellets were air dried and re-suspended in TE. The dissolved nucleic acids were brought to 1.4M NaCl and re-precipitated using 2 volumes of 75 % ethanol(If the pellet obtained was hard to re-suspend, this step was repeated one more time. Also, when colour DNA pellet was obtained, the colour can be removed using 2-3 extractions with ethanol.). The pellets were washed twice using 100 % ethanol**4**, dried and re-suspended in 100 µl of TE buffer. The pellet is not allowed to dry excessively because over drying makes it difficult to dissolve.

The tube was incubated at 37°C for 30 min to dissolve genomic DNA, and RNase was then added.

Amount and Purity of DNA
The yield of DNA per gram of leaf tissue extracted was measured using a UV-VIS Spectronic 5 (Milton Roy) spectrophotometer at 370 nm. The purity of DNA was determined by calculating the ratio of absorbance at 370 nm to that of 480 nm. DNA samples from the fruit tissues were digested with Sau3A and electrophoresed on a 0.8% Agarose gel, according to Sam brook *et al.* (1989).

PCR reactions and electrophoresis
The primer used was (GACA) 5:5'GACAGACAGACAGACAGACA-3'. Specific annealing temperature (Ta) determined (GACA) 5 was 62 °C. PCR reactions were performed with the Gene Amp PCR System 2400 Perkin Elmer. The PCR conditions were optimised for other thermo-circlers and annealing temperatures was optimised for each primer set.

Each 25 -l reaction volume contains 2.5 -l reaction buffer (10x), 2.5 _l MgCl2 (25 mM), 2 -l dNTP mixture (2.5 mM), 4 -l of primer (10- mol l-1), 0.5 -l Taq DNA polymerase (Red Gold star™ DNA polymerase, Eurogentec, 5 units/-1) and 1 -l of DNA (40 ng). PCR consists of one cycle of 94 °C, 2 min, which was followed by 27 cycles of 94 °C, 1 min; 62 °C , 1 min; 72 °C, 2 min, and finally one cycle of 72°C, 7 min. The PCR products were analyzed by electrophoresis using a 2 % agarose gel in TBE buffer. DNA was stained by soaking the gel in a 0.5 mg/ml ethidium bromide solution.

RESULTS AND DISCUSSION
Figure 1: (a) Electrophoresis of fruit DNA on 0.8 % Agarose gel following RNase treatment. Lanes 1-3: unripe DNA fruit; 4 µl DNA was loaded per lane.

Figure 1: (a) Electrophoresis of fruits DNA on 0.8% Agarose Gel following RNase treatment. Lanes 1-3: fruits DNA fruit. 4 µl DNA was loaded per lane.

We first investigated the effect of detergents in the DNA extraction buffer. Detergents, SDS and CTAB, were added to the solution containing 100 mM Tris-HCl (pH 8.0), 1.4 M NaCl, 50 mM EDTA, and 1 % - mercaptoethanol. During the addition of preheated CTAB containing -mercaptoethanol, moving quickly at this stage was critical in getting good quality DNA.

To help in minimizing time spent doing this step, the 1 ml of 2 % CTAB was measured in a 2 ml micro centrifuge tube to which 100 µl of -mercaptoethanol (1 %, v/v) was added and the tube placed in a 60 °C water bath until ready for use. Addition of the pre-warmed, pre-measured CTAB buffer to the frozen leaf tissue contained in the pre-chilled conical tube saves precious time in bringing the tissue from -80 °C to 60 °C as rapidly as possible resulting in DNA of higher quality (Puchooa, 2004). Using 1% -mercaptoethanol produced nucleic acid pellets that were not nearly brown. Inclusion of PVP improved the colour of the nucleic acid obtained. DNA could only be extracted with the solution containing CTAB. The addition of -mercaptoethanol to the CTAB extraction buffer prior to incubation is also a critical factor (Figure 1a). The purity of genomic DNA was dependent on the number of washes. A three-time wash combined with a short-run centrifugation was sufficient for DNA purification and removal of endogenous nucleases or other proteins. As CTAB is soluble in ethanol, residual amounts are removed in the subsequent wash. During ethanol precipitation of nucleic acids from 1.4 M NaCl, polysaccharides remain dissolved in the ethanol (Fang *et al*., 1992). The freer the nucleic acids are from contaminants, the easier it is to re-suspend the pellet. If the pellet obtained from the first ethanol precipitation from 1.4 M NaCl was found to be hard to re-suspend, two such precipitations were done and the pellet obtained from the second precipitation usually goes into solution very easily. It was found that washing in 80 % ethanol gave better DNA as a result of the removal of any residual NaCl and/or CTAB. The DNA extracted can be digested with restriction enzymes such as Sau3A (Figure 1b).

Figure 1: (b) Restriction enzymes digestion of *Annona senegalensis* genomicDNA. Lanes 1-3: DNA digested with Sau3A.

DNA quality was estimated by measuring the 360/480 UV absorbance ratio which varied between 1.8 and 2. In only a few samples with extremely low DNA contents was the ratio lower than 1.8.We evaluated the quality of the extracted DNA through two procedures: Agarose gel electrophoresis and SSRPCR. Figure 1 shows the result of the extracted DNA run on a 0.8% Agarose gel, stained with ethidium bromide and visualized with UV light. In order to check the efficiency and reliability of the method, we first amplified the DNA of ripped and unripe fruits using the primer, (GACA) 5. The amplified PCR products of leaf DNA showed identical band patterns and similar intensity to that of leaf tissue. However,

different PCR patterns were obtained between the fruits (Figure 2). We performed SSR-PCR amplification tests on all samples using primer and protocols previously optimized in the agarose gel. Figure 2 shows amplification products from *Annona* fruits.

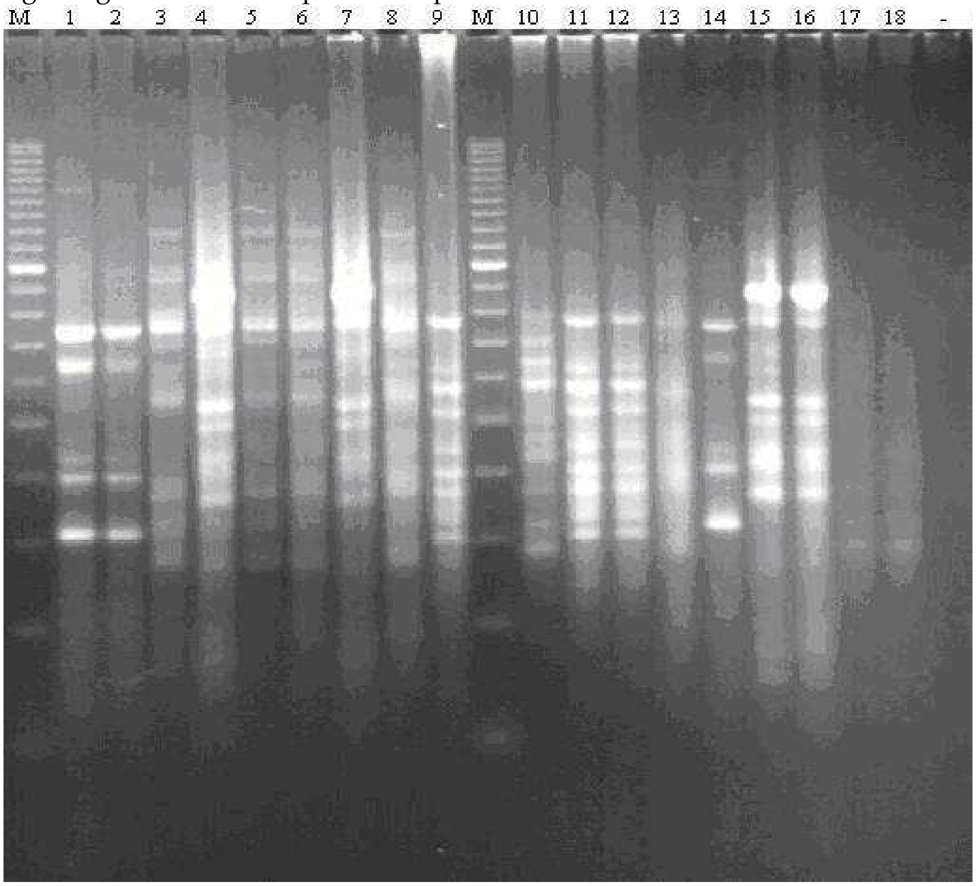

Figure 2. Amplification of purified DNA with SSR-PCR. DNA was purified using the method described. The purified DNA was amplified using SSR-PCR and the amplification products were separated on a 2% agarose gel, stained with ethidium bromide and visualized with UV light. Lanes 1-18: *Annona* fruits amplified using SSR-PCR primer (GACA) 5, for reference, a negative control (-) was included. Lane M: contains a 100 bp DNA size marker.

CONCLUSION

DNA purification from plant leaves has become the bottleneck in sample processing from plant tissue to PCR result. This procedure can be used to purify high-quality DNA from plant material using a walkway protocol. Purified DNA performed well in SSR-PCR and gave good yield. This will allow plant molecular biologists to achieve increased productivity when purifying plant genomic DNA in low to moderate throughput systems.

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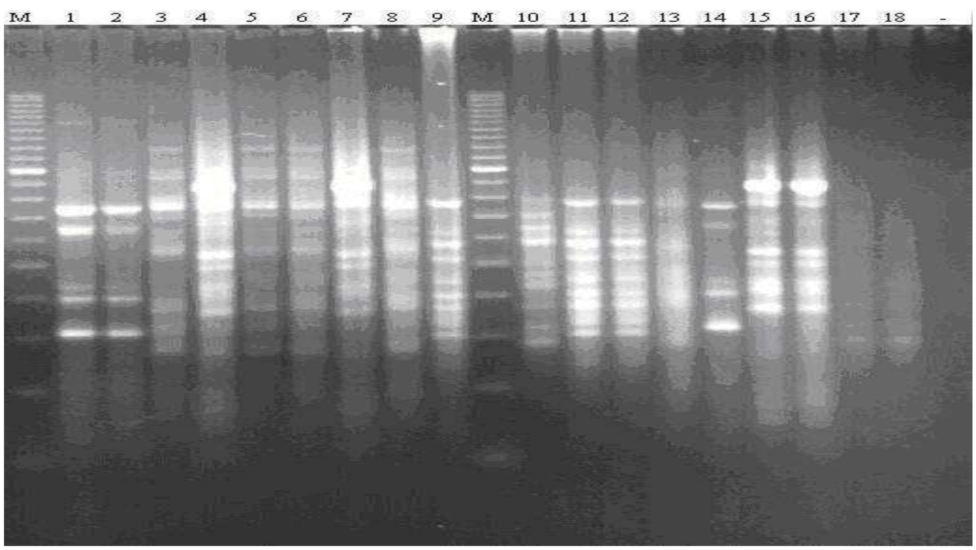

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CHAPTER 8

FOOD PROCESSING- PROSPECTS IN MODERN TIMES AND CHALLENGES AHEAD

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ABSTRACT

Food processing involves the conversion of raw ingredients into more acceptable food forms. Food processing is related to crops after harvesting, animal products prepared after slaughtering of animals and converting these products to appeal the general consumers for market profitability and for increasing the storage life of the finished processed products. Animal and fish feeds are also manufactured by this same mechanism of processing.

KEYWORDS: Food processing, raw ingredients, processed products

INTRODUCTION

There are several advantages of proper food processing under controlled and regulated conditions. It implies the decrease or removal of the content of anti-nutritional factors from the food, increase in shelf-life for prolonged preservation, ease in marketing and increase in consumer demands and increment in the quality and consistency of the finished processed food. It also increases the availability of many food items during off-seasons, increases the convenience in transportation of food items over long distances by decreasing the chances of rotting of mainly perishable food items and increasing the safety for consumption by deleting pathogenic microorganisms which cause spoilage. Food processing at certain places can also be used to reduce the conditions of food shortage and by supplementation of nutritious and safe food for the masses (Laudan 2010a,b).

BENEFITS AND NEED IN MODERN TIMES

As food processing decreases the population or load of pathogenic microorganisms in food and neutralizes the harmful mycotoxins, if present therein. So, it reduces the chances of food-borne diseases caused by microorganisms like Salmonella etc. which can harbor in raw meat and incidences of mycotoxicoses (majorly, aflatoxicosis, ochratoxicosis and zearalenone) due to prolonged improper storage of food thereby causing human illnesses (Ganguly, 2012). Food processing has also gained its importance in the wide variety of diet among people throughout the globe and availability of exotic food items at various places. Processing of food items enhance the taste, flavor and aroma of the food thereby increasing the overall chances of its acceptability among the masses (Laudan 2010b).

Food processing whenever performed in large mass is comparatively cheaper than processing and modification of individual ingredients. So, the food processing sector implies a huge margin of profit for processed food manufacturers and retailers in the supply chain.

Processing involves various methods among which cooking is a very popular and widely used method which involves the modification by blending etc. of naturally available unprocessed food ingredients. In our nowadays fast paced lifestyle where every family member is on a go for financial security, processed food products have gained its important

position in daily livelihood by offering ready prepared wholesome and nutritious meals within short period (Laudan 2010a).

The modern methods of processing decreases the risk of health hazards to consumers from diabetics, allergies etc. Food processing also involves fortification for the production of neutraceuticals and energy supplements with addition of probiotics, prebiotics, certain important vitamins and mineral elements within standard permissible limits which are rather present in natural food in very scarce quantity.

DRAWBACKS OF FOOD PROCESSING

There exist certain limitations of food processing also. For example, during processing by heating the concentration of vitamin C is reduced, as it is heat-sensitive. Generally, food processing techniques reduce the nutritional quantity in very negligible amount of nearly 5-20%. Food processing involves the use of food additives, which sometimes prove to be detrimental to public health. For this reason, the European Food Safety Authority (EFSA) [Regulation No.178/2002] has specified the level of individual feed additive during processing technique and which is approved for safe consumption of human beings. The additives after approval gain an 'E' number (E stand for Europe) which signifies the quantity of the additive to be incorporated in the finished processed food item.

Food processing involves many mechanisms like mixing, grinding, chopping and emulsifying during the whole process of production, which indirectly increase the chances of contamination and admixtures with undesirable foreign elements. Sometimes, packaging containers also pose a threat for public contamination when exposed to thorough procedures of continuous processing by leaching of the chemical components from the containers into the food item to be processed.

In food manufacturing practices, using metal detectors decrease the risk of contamination with metal fragments during the processing technique. In large food processing equipments are fitted with many metal detectors at several positions to negate the chances and risks of metal contamination of processed food products. In 1947, the first industrial purpose metal detector was introduced by Goring Kerr.

REGULATORY PARAMETERS FOR GOVERNING PROCESSING EFFICIENCY

Processing techniques can be lengthy and time consuming sometimes depending on the type of food being processed and it needs the control and regulation of certain parameters for processing which includes hygiene which is assessed by the microbial load in the processed food product, efficiency in energy utilization, minimum waste generation, effective labour saving and minimization of cleaning requirements. Hygiene protocols for the finished processed food product are evaluated as per HACCP guidelines (FAO Reports 2007a,b,c; FAO Report 2010) to minimize the risk of potential health hazards among consumers. Baking is nowadays a more preferable technique of food processing rather than frying on grounds of long-term health benefits and retaining the natural taste and flavor of the finished product. Use of artificial sweeteners and leavening agents also impose long-term serious health risks to regular consumers by acting as diabetics.

The popular processing techniques in food sector are canning, fish processing, industrial rendering, tanneries, meat packing plants, slaughter houses, sugar industries and vegetable packaging plants.

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CHAPTER 9

ENZYMES FOR GENETIC ENGINEERING

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Genetic engineering is the direct manipulation of an organism's genome using biotechnology. New DNA may be inserted in the host genome by first isolating and copying the genetic material of interest using molecular cloning methods to generate a DNA sequence, or by synthesizing the DNA, and then inserting this construct into the host organism. Genetic engineering techniques have been applied in numerous fields including research, agriculture, industrial biotechnology, and medicine. Genetic engineering is a broad term referring to manipulation of an organisms' nucleic acid.

Organisms whose genes have been artificially altered for a desired affect is often called genetically modified organism (GMO). Recombinant DNA technology (rDNA) is technology that is used to cut a known DNA sequence from one organism and introduce it into another organism thereby altering the genotype (hence the phenotype) of the recipient. The process of introducing the foreign gene into another organism (or vector) is also called cloning. Sometimes these two terms are used synonymously.

The basic events involved in genetic engineering includes –

- Isolation of gene of interest
- Gene of interest to be cloned incorporated into a vector
- The recombinant vector (r DNA) is introduced into a host cell by transformation
- Selection of transformed cells
- rDNA molecule is multiplied within the host cell to produce number of identical copies

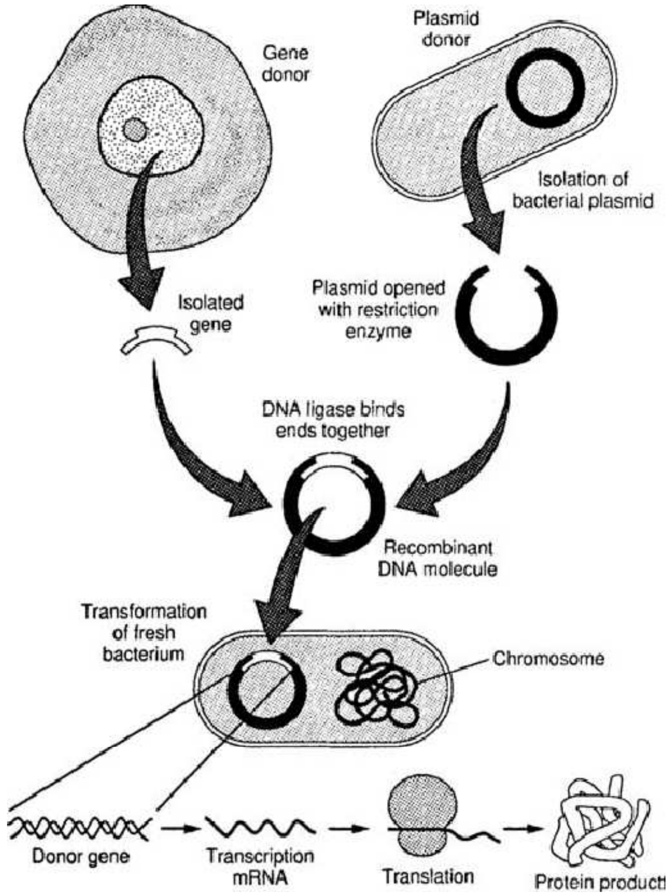

Figure 1- Basic steps involved in genetic engineering

Genetic engineering techniques are used to achieve the following-

- Study the arrangement, expression and regulation of genes
- Modification of genes to obtain a changed protein product
- Modification of gene expression either to enhance or suppress a particular product
- Making multiple copies of a nucleic acid segment artificially
- Introduction of genes from organism to another, thus creating a transgenic organism
- Creation of organism with desirable or altered characteristics

In the cloning or genetic engineering various purified enzymes are used to cut, ligate or modify the DNA. Such enzymes which are used to create recombinant DNA molecule termed as DNA tailoring enzymes. The important enzymes which are discussed in the presented chapter are as follow-

1. Restriction endonucleases
2. Reverse transcriptase
3. Ribonuclease-H
4. SI nuclease
5. Taq DNA polymerase
6. Terminal nucleotidial transferase
7. Alkaline phosphatase
8. Polynucleotide kinase
9. DNA ligase

DNA cutting enzymes may be exonucleases which are enzymes that work by cleaving nucleotides from the end (exo) of a polynucleotide chain. A hydrolyzing reaction that breaks phosphodiester bonds at either the 3′ or the 5′ end occurs whereas,endonucleases are enzymes that cleave the phosphodiester bond within a polynucleotide chain.

1. Restriction endonucleases

The term restriction enzyme originated from the studies of phage λ and the phenomenon of host-controlled restriction and modification of a bacterial virus. Restriction endonucleases are components of restriction modification (RM) systems that occur ubiquitously among bacteria, archaea and in viruses of certain unicellular algae. The phenomenon was first identified in work done in the laboratories of Salvador Luria and Giuseppe Bertani in early 1950s. It was found that a bacteriophage λ that can grow well in one strain of *Escherichia coli*, for example*E. coli* C, when grown in another strain, for example *E. coli* K, its yields can drop significantly, by as much as 3-5 orders of magnitude. The *E. coli* K host cell, known as the restricting host, appears to have the ability to reduce the biological activity of the phage λ. If a phage becomes established in one strain, the ability of that phage to grow also becomes restricted in other strains. In the 1960s, it was shown in work done in the laboratories of Werner Arber and Matthew Meselson that the restriction is caused by an enzymatic cleavage of the phage DNA, and the enzyme involved was therefore termed a restriction enzyme.

The restriction enzymes studied by Arber and Meselson were type I restriction enzymes which cleave DNA randomly away from the recognition site. In 1970, Hamilton O. Smith, Thomas Kellyand Kent Welcox isolated and characterized the first type II restriction enzyme, Hind II, from the bacterium *Haemophilus influenzae*. This type of restriction enzymes is more useful for laboratory use as they cleave DNA at the site of their recognition sequence. For their work in the discovery and characterization of restriction enzymes, the 1978 Nobel Prize for Physiology or Medicine was awarded to Werner Arber, Daniel Nathans, and Hamilton O. Smith. These enzymes occur naturally in bacteria as a chemical weapon against the invading viruses and cut both strands of DNA when certain foreign nucleotides are introduced in the cell. These enzymes cleave a DNA to generate a nick with a 5' phosphoryl and 3' hydroxyl termini. Restriction enzymes probably evolved to provide a defense mechanism against invading viruses. Inside a bacterium, the restriction enzymes selectively cut up foreign DNA in a process called restriction while host DNA is protected by a modification enzyme (a methylase) that modifies the bacterial DNA and blocks cleavage. Together, these two processes form the restriction modification (RM) system.

Restriction enzymes recognize a specific sequence of nucleotides and produce a double-stranded cut in the DNA. The recognition sequences usually vary between 4 and 8 nucleotides, and many of them are palindromic sequence, meaning the base sequence reads the same backwards and forwards. There are two types of palindromic sequences that can be possible in DNA. The mirror-like palindrome is similar to those found in ordinary text, in which a sequence reads the same forward and backwards on a single strand of DNA strand, as in GTAATG. The inverted repeat palindrome is also a sequence that reads the same forward and backwards, but the forward and backward sequences are found in complementary DNA strands (i.e., of double-stranded DNA), as in GTATAC (GTATAC being complementary to CATATG). Inverted repeat palindromes are more common and

have greater biological importance than mirror-like palindromes. Restriction enzyme may generate sticky ends (eg. EcoRI) as well as blunt ends (eg. SmaI)

Sticky ends Blunt ends

Recognition sequences in DNA differ for each restriction enzyme, producing differences in the length, sequence and strand orientation (5' end or the 3' end) of a sticky-end "overhang" of an enzyme restriction. Different restriction enzymes that recognize the same sequence are known as neoschizomers. These often cleave in different locales of the sequence. Different enzymes that recognize and cleave in the same location are known as isoschizomers.

Nomenclature of restriction enzymes

The restriction enzymes are named based on the following principles:

1 Name of the organism is identified by the first letter of the genus name and the first two letters of the species name to form a three letter abbreviation in the italic, for example, *E. coli* = *Eco* and *H. influenzae* = *Hin,* etc.
2. A strain or type identified is written as subscript *e.g.* *EcoK* for *E. coli* strainK, *Hind* for *H. influenzae* strain Rd.
3. In such cases where the restriction and modification systems are genetically specified by a virus or plasmid, the extra chromosomal element is identified by a subscript *e.g. Eco* RI, *Eco*PI, etc.
4. When a strain has several restriction and modification systems, these are identified by Roman numerals, for example *Hindi, Hindll, Hindlll* for *H. influenzae* strain Rd. etc. These Roman numerals should not be confused with those in the classification of restriction enzymes into Type I.

Types of restriction enzymes

Naturally occurring restriction endonucleases are categorized into three groups (Types I, II and III) based on their composition and enzyme cofactor requirements, the nature of their target sequence, and the position of their DNA cleavage site relative to the target sequence. All types of enzymes recognize specific short DNA sequences and carry out the endonucleolytic cleavage of DNA to give specific fragments with terminal 5'-phosphates. They differ in their recognition sequence, subunit composition, cleavage position, and cofactor requirements.

Type I Restriction endonuclease (EC 3.1.21.3)

Type I restriction enzymes were the first to be identified and were first identified in two different strains (K-12 and B) of *E. coli*. These enzymes cut at a site that differs, and is a random distance (at least 1000 bp) away, from their recognition site. Cleavage at these random sites follows a process of DNA translocation, which shows that these enzymes are also molecular motors. The recognition site is asymmetrical and is composed of two specific portions—one containing 3–4 nucleotides, and another containing 4–5 nucleotides—separated by a non-specific spacer of about 6–8 nucleotides. These enzymes are multifunctional and are capable of both restriction and modification activities, depending upon the methylation status of the target DNA. The cofactors S-Adenosyl methionine , hydrolyzed adenosine triphosphate (ATP), and magnesium (Mg^{2+}) ions, are required for their full activity. Type I restriction enzymes possess three subunits called HsdR, HsdM, and

HsdS; HsdR is required for restriction; HsdM is necessary for adding methyl groups to host DNA (methyltransferase activity) and HsdS is important for specificity of the recognition (DNA-binding) site in addition to both restriction (DNA cleavage) and modification (DNA methyltransferase) activity. Typical examples are EcoKI, EcoAI, EcoR124I and StySBLI, which represent Type IA, IB, IC and ID subtypes, respectively.

Type II Restriction endonuclease (EC 3.1.21.4)

Typical type II restriction enzymes differ from type I restriction enzymes in several ways. They are a homodimer, with recognition sites are usually undivided and palindromic and 4–8 nucleotides in length. They recognize and cleave DNA at the same site, and they do not use ATP or S-Adenosyl methionine for their activity—they usually require only Mg^{2+} as a cofactor. These are the most commonly available and used restriction enzymes. The DNA fragments produced have 'blunt' or 'sticky' ends with 3'- or 5'-overhangs of up to 5 nucleotides (there is a single known example of an enzyme producing a 7-nucleotide 3'-overhang: TspRI (CASTGNN/)). Most of the restriction enzymes used for recombinant DNA work belong to this subtype, which is called Type IIP (P for –palindromic) according to the accepted nomenclature. Many Type II restriction endonucleases have properties different from the Type IIP enzymes, for which EcoRI (recognition sequence G/AATTC) and EcoRV (GAT/ATC) are the best-known and best-studied representatives. The current nomenclature tries to group the Type II restriction enzymes according to properties that are unique to the respective subtype. However, as will be seen, overlap cannot be avoided. This is the consequence of the great diversity among Type II restriction endonucleases.

To bind to DNA restriction enzymes have to 'open' their DNA binding site. In several cases, however, structural information of the free enzyme implies that the DNA binding site does not appear to be sufficiently open to allow DNA binding, whereas in most instances the enzymes make their binding sites accessible in a 'tonglike' motion, which is perpendicular to the DNA axis (*e.g.* BamHI, EcoRV, PvuII), BglII uses a 'scissor-like' motion, which is parallel to the DNA axis. The question arises whether restriction enzymes oscillate between closed and open states, or whether the open state is induced by association of enzyme with DNA. For EcoRV it was demonstrated that there is an 'external binding' site, which when occupied by DNA may open the gate of the 'inner'DNA binding site. All restriction endonucleases face the problem of efficiently finding their specific site in the presence of a huge excess of non-specific sites, to which they can also bind although with a much lower affinity. EcoRI was the first restriction enzyme for which evidence was presented that it makes use of facilitated diffusion for target site location. Facilitated diffusion is a very effective process that not only speeds up target site location by a factor > 10, but also increases the processivity of restriction endonucleases and accelerates the dissociation from the specific site after cleavage. Under optimum conditions restriction endonucleases can scan ~106 bp in one binding event; due to the random movement on the DNA, the effective distance scanned is ~1000 bp. Three principally different, but not mutually exclusive, mechanisms can account for the efficiency of target site location by DNA-binding proteins: (i) 'sliding'; (ii) 'jumping' or 'hopping'; and (iii) intersegment transfer.

Phosphodiester bond hydrolysis by Type II restriction endonucleases follows an SN2-type mechanism, which is characterized by inversion of configuration at phosphorous. The general mechanism of phosphodiester hydrolysis comprises three steps: (i) the preparation of the attacking nucleophile by deprotonation, (ii) the nucleophilic attack of the hydroxide ion on the phosphorous leading to the formation of the pentavalent transition state, To

achieve efficient catalysis, all three steps require an assisting group: (i) a base to deprotonate the water molecule; ii) a Lewis acid that stabilizes the pentavalent transition state with two negative charges; and (iii) an acid that protonates the leaving 3' oxyanion.

Type III Restriction endonuclease (EC 3.1.21.5)

Type III restriction enzymes recognize two separate non-palindromic sequences that are inversely oriented. They cut DNA about 20-30 base pairs after the recognition site. These enzymes contain more than one subunit and require S-Adenosyl methionine and ATP cofactors for their roles in DNA methylation and restriction, respectively. They are components of prokaryotic DNA restriction-modification mechanisms that protect the organism against invading foreign DNA. Type III enzymes are hetero-oligomeric, multifunctional proteins composed of two subunits, Res and Mod. The Mod subunit recognises the DNA sequence specific for the system and is a modification methyltransferase as such it is functionally equivalent to the M and S subunits of type I restriction endonuclease.

Type III enzymes recognise short 5-6 bp long asymmetric DNA sequences and cleave 25-27 bp downstream to leave short, single-stranded 5' protrusions. They require the presence of two inversely oriented unmethylated recognition sites for restriction to occur. These enzymes methylate only one strand of the DNA, at the N-6 position of adenosyl residues, so newly replicated DNA will have only one strand methylated, which is sufficient to protect against restriction.

Star activity of restriction enzymes

Star activity is a relaxation or alteration of the specificity of restriction enzyme mediated cleavage of DNA that can occur under reaction conditions that differ significantly from those optimum for the enzyme. The result is typically cleavage at non-canonical recognition site, or sometimes complete loss of specificity. Differences which can lead to star activity include low ionic strength, high pH, and high (> 5% v/v) glycerol concentrations. The latter condition is of particular practical interest, since commercial restriction enzymes are usually supplied in a buffer containing a substantial amount of glycerol (50% v/v is typical), meaning insufficient dilution of the enzyme solution can cause star activity; this problem most often arises during double or multiple digests.

2. Reverse Transcriptase

Reverse transcriptase, also called RNA-directed DNA polymerase, an enzyme encoded from the genetic material of retroviruses that catalyzes the transcription of retrovirus RNA (ribonucleic acid) into DNA (deoxyribonucleic acid). This catalyzed transcription is the reverse process of normal cellular transcription of DNA into RNA, hence the names reverse transcriptase and retrovirus.

In 1970 Temin and Japanese virologist Satoshi Mizutani, and American virologist David Baltimore, working independently, reported the discovery of an enzyme that could synthesize proviral DNA from the RNA genome of RSV. This enzyme was named RNA-directed DNA polymerase, commonly referred to as reverse transcriptase. This discovery resulted in the identification of a unique virus family (Retroviridae), and the understanding of the pathogenesis of these viruses spurred a rush to discover other infectious cancer-causing agents. In 1975 Temin, Baltimore, and Dulbecco (who mentored both Temin and

Baltimore) were awarded the Nobel Prize for Physiology or Medicine for their discoveries concerning the interaction between tumour viruses and the genetic material of the cell.

Reverse transcriptase is central to the infectious nature of retroviruses, several of which cause disease in humans, including human immunodeficiency virus (HIV), which causes acquired immunodeficiency syndrome (AIDS), and human T-cell lymphotrophic virus I (HTLV-I), which causes leukemia. Reverse transcriptase is also a fundamental component of a laboratory technology known as reverse transcription-polymerase chain reaction (RT-PCR), a powerful tool used in research and in the diagnosis of diseases such as cancer.

Retroviruses consist of an RNA genome contained within a protein shell that is enclosed in a lipid envelope. The retrovirus genome is typically made up of three genes: the group-specific antigen gene (gag), the polymerase gene (pol), and the envelope gene (env). The pol gene encodes the three enzymes—protease, reverse transcriptase, and integrase—that catalyze the steps of retroviral infection. Once a retrovirus is inside a host cell (a process mediated by protease), it takes over the host's genetic transcription machinery to construct a DNA provirus. This process, the conversion of retroviral RNA to proviral DNA, is catalyzed by reverse transcriptase and is necessary for proviral DNA insertion into host DNA—a step initiated by the integrase enzyme.

3. Ribonuclease H (RNase H)

The enzyme RNase H is a non-specific endonuclease and catalyzes the cleavage of RNA via a hydrolytic mechanism. Members of the RNase H family can be found in nearly all organisms, from archaea to bacteria and eukaryota. RNase H's ribonuclease activity cleaves the 3′-O-P bond of RNA in a DNA/RNA duplex to produce 3′-hydroxyl and 5′-phosphate terminated products. In DNA replication, RNase H is responsible for removing the RNA primer, allowing completion of the newly synthesized DNA. There are two main types of RNase H, and at least one of them is present in most organisms.

Eukaryotic RNases H are larger and more complex than their prokaryotic counterparts. Eukaryotic RNase H1 has acquired a hybrid binding domain that confers processivity and affinity for the substrate, whereas eukaryotic RNase H2 is composed of three different proteins: the catalytic subunit (2A), similar to the monomeric prokaryotic RNase HII, and two other subunits (2B and 2C) that have no prokaryotic counterparts and as yet unknown functions, but that are necessary for catalysis. In a molecular biology laboratory, as RNase H specifically degrades the RNA in RNA:DNA hybrids and will not degrade DNA or unhybridized RNA, it is commonly used to destroy the RNA template after first-strand complementary DNA (cDNA) synthesis by reverse transcription, as well as procedures such as nuclease protection assays.

RNase H can also be used to degrade specific RNA strands when the cDNA oligo is hybridized, such as the removal of the poly (A) tail from mRNA hybridized to oligo (dT), or the destruction of a chosen non-coding RNA inside or outside the living cell. To terminate the reaction, a chelator, such as EDTA, is often added to sequester the required metal ions in the reaction mixture. Prokayotes and some single-cell eukaryotes do not require RNases H for viability, in higher eukaryotes RNases H are essential. Mutations in any of the three subunits of human RNase H2 cause Aicardi-Goutières syndrome, a human neurological disorder with devastating consequences.

4. SI nuclease
SI nuclease is an enzyme that selectively cuts and degrades single stranded portion of DNA. It is a glycoprotein consisting of 82%glycoprotein and 18%carbohydrates unit. The molecular weight of the enzyme is 38,000 Daltons. This enzyme breaks the phosphodiester bond between two nucleotides in single stranded portion of DNA and then degrades single stranded extensions. It does not degrade double stranded portions of DNA and RNA.

SI nuclease is used to degrade the hairpin loop formed while making a duplex DNA from complementary DNA strand and also used to remove unwanted tail sequence from DNA fragment to make them blunt ends. It is used to remove the extra adenine base from DNAs synthesized as a result of polymerase chain reaction.

5. Taq DNA polymerase
Taq polymerase is a thermo-stable DNA polymerase named after the thermophilic bacterium *Thermus aquaticus* from which it was originally isolated by Thomas D. Brock in 1965. It is frequently used in polymerase chain reaction (PCR), a method for greatly amplifying short segments of DNA. *T. aquaticus* is a bacterium that lives in hot springs and hydrothermal vents, and Taq polymerase was identified as an enzyme able to withstand the protein-denaturing conditions (high temperature) required during PCR. Therefore it replaced the DNA polymerase from E. coli originally used in PCR. Taq's optimum temperature for activity is 75–80°C, with a half-life of greater than 2 hours at 92.5°C, 40 minutes at 95°C and 9 minutes at 97.5°C, and can replicate a 1000 base pair strand of DNA in less than 10 seconds at 72°C.

One of Taq's drawbacks is its relatively low replication fidelity. It lacks a 3' to 5' exonuclease proofreading activity, and has an error rate measured at about 1 in 9,000 nucleotides. The remaining two domains however may act in coordination, via coupled domain motion. Some thermostable DNA polymerases have been isolated from other thermophilic bacteria and archaea, such as Pfu DNA polymerase, possessing a proofreading activity, and are being used instead of (or in combination with) Taq for high-fidelity amplification.

Taq makes DNA products that have A (adenine) overhangs at their 3' ends. This may be useful in TA cloning, whereby a cloning vector (such as a plasmid) that has a T (thymine) 3' overhang is used, which complements with the A overhang of the PCR product, thus enabling ligation of the PCR product into the plasmid vector.

6. Terminal nucleotidial transferase
Terminal nucleotidial transferase or Terminal deoxynucleotidyl transferase (TdT), also known as DNA nucleotidylexotransferase (DNTT), is a specialized DNA polymerase expressed in immature, pre-B, pre-T lymphoid cells, and acute lymphoblastic leukemia/lymphoma cells. TdT adds N-nucleotides to the V, D and J exons during antibody gene recombination, enabling the phenomenon of junctional diversity. In humans, terminal transferase is encoded by the DNTT gene.

TdT catalyses the addition of nucleotides to the 3' terminus of a DNA molecule. Unlike most DNA polymerases it does not require a template. The preferred substrate of this enzyme is a 3'-overhang, but it can also add nucleotides to blunt or recessed 3' ends. Cobalt is a necessary cofactor, however the enzyme catalyzes reaction upon Mg and Mn administration in vitro.

Terminal Deoxynucleotidyl Transferase (TdT), a template-independent DNA polymerase, catalyzes the repetitive addition of deoxyribonucleotides to the 3'-OH of oligodeoxyribonucleotides and single-stranded or double-stranded DNA. The TdT requires an oligonucleotide of at least three nucleotides to serve as a primer. With RNA as template TdT shows variable performance which strongly depends upon the tertiary structure of acceptor RNA 3′-end and the nature of nucleotide. Generally, it is lower than using DNA as a template.

Purified TdT enzymes used for the Production of synthetic homo- and heteropolymers, homopolymeric tailing of linear duplex DNA with any type of 3'-OH terminus, oligodeoxyribonucleotide and DNA labeling, 5'-RACE (Rapid Amplification of cDNA Ends, in- situ localization of apoptosis.

7. Alkaline phosphatase

Alkaline phosphatase digests the terminal phosphate group at the 5' end of a DNA fragment. It acts on both the DNA and RNA. The enzyme consist of two identical sub-units, and has the molecular weight of 140,000 Daltons. There are four zinc atom in an alkaline phosphatase molecule. Alkaline phosphatase is used to remove the 5' end phosphate group from the linearised vector DNA. For labeling DNA and RNA, radioactive p32 is added to the 5' end of the DNA. If a phosphate group is present at the 5' end, cannot add p32 to the DNA. So the 5'-phosphate group is removed before adding radioactive p32 to the DNA by using alkaline phosphatase.

8. Polynucleotide kinase

Polynucleotide kinase (or PNK) is a T7 bacteriophage (or T4 bacteriophage) enzyme that catalyzes the transfer of a gamma-phosphate from ATP to the free hydroxyl end of the 5' DNA or RNA. The resulting product could be used to end-label DNA or RNA, or in a ligation reaction. Polynucleotide kinase is used to re-phosphorylate vector DNA in rDNA. Then only DNA ligase can seal the nick between the vector DNA and target DNA (desired DNA). It is also used to transfer radioactive p32 from ATP to dephosphorylated 5' end of DNA or RNA for labeling. The labeling techniques is used to make hybridization probe, diagnostic kits, analyse the base sequence of DNA and to construct restriction maps.

9. DNA ligase

In molecular biology, DNA ligase is a specific type of enzyme, a ligase, that facilitates the joining of DNA strands together by catalyzing the formation of a phosphodiester bond. It plays a role in repairing single-strand breaks in duplex DNA in living organisms, but some forms (such as DNA ligase IV) may specifically repair double-strand breaks (i.e. a break in both complementary strands of DNA). Single-strand breaks are repaired by DNA ligase using the complementary strand of the double helix as a template, with DNA ligase creating the final phosphodiester bond to fully repair the DNA.

DNA ligase has applications in both DNA repair and DNA replication. In addition, DNA ligase has extensive use in molecular biology laboratories for genetic recombination experiments. Purified DNA ligase is used in gene cloning to join DNA molecules together to form recombinant DNA.

The mechanism of DNA ligase is to form two covalent phosphodiester bonds between 3' hydroxyl ends of one nucleotide, ("acceptor") with the 5' phosphate end of another ("donor"). ATP is required for the ligase reaction, which proceeds in three steps:

(a) Adenylation (addition of AMP) of a residue in the active center of the enzyme, pyrophosphate is released
(b) Transfer of the AMP to the 5' phosphate of the so-called donor, formation of a pyrophosphate bond
(c) Formation of a phosphodiester bond between the 5' phosphate of the donor and the 3' hydroxyl of the acceptor

DNA ligases have become an indispensable tool in modern molecular biology research for generating recombinant DNA sequences. For example, DNA ligases are used with restriction enzymes to insert DNA fragments, often genes, into plasmids. One vital aspect to performing efficient recombination experiments involving the ligation of cohesive-ended fragments is controlling the optimal temperature. Most experiments use T4 DNA Ligase (isolated from bacteriophage T4), which is most active at 25°C. However, for optimal ligation efficiency with cohesive-ended fragments ("sticky ends"), the optimal enzyme temperature needs to be balanced with the melting temperature Tm (also the annealing temperature) of the sticky ends being ligated. If the ambient temperature exceeds Tm, the homologous pairing of the sticky ends would not be stable because the high temperature disrupts hydrogen bonding. Ligation reaction is most efficient when the sticky ends are already stably annealed, disruption of the annealing ends would therefore results in low ligation efficiency. The shorter the overhang, the lower the Tm, typically a 4-base overhang has a Tm of 12-16°C.

Since blunt-ended DNA fragments have no cohesive ends to anneal, the melting temperature is not a factor to consider within the normal temperature range of the ligation reaction. However, the higher the temperature, the less chance that the ends to be joined will be aligned to allow ligation (molecules move around the solution more at higher temperatures). The limiting factor in blunt end ligation is not the activity of the ligase but rather the number of alignments between DNA fragment ends that occur. The most efficient ligation temperature for blunt-ended DNA would therefore be the temperature at which the greatest number of alignments can occur. Therefore, the majority of blunt-ended ligations are carried out at 14-20°C overnight. The absence of a stably annealed ends also means that the ligation efficiency is lowered, requiring a higher ligase concentration to be used.

CHAPTER 10

UTILIZATION OF AGRO-INDUSTRIAL RESIDUES AS SUBSTRATE OF SOLID-STATE FERMENTATION

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ABSTRACT
In recent years, cultural and technological advances have stimulated the expansion in agricultural production as well as development of new products and improvement of quality in all aspects of the agro-industry. As a consequence of the increase in productivity, high quantities of waste have been generated and, without a proper destination, this waste can result in a major damage to the environment. Due to interesting features of these residues many studies have been performed to transform them into new products or even use them as a substrate for processes such as solid-state fermentation. The advantage of its use is generating products in large quantities with lower production costs, such as enzymes, organic acids, antibiotics, biomass and others. The employ of solid-state fermentation has been increasing, mainly due to the characteristics of ease application and the adaptation of used microorganisms. However, several extrinsic and intrinsic factors must be taken into consideration for the development and maintenance of solid-state fermentation, as well as its income.

KEYWORDS: Agriculture; by-products; solid-state fermentation; environment.

RURAL DEVELOPMENT

The global agricultural production is growing, mainly led by Brazil, Russia, India, Ukraine, China and USA. Despite the biggest producers, large amounts derive from small farms. Only in Africa there are about 33 million small farms in an area of 300 million hectares of arable land. In Asia, China has nearly half of the world's small farms in an area of 193 million hectares. On a smaller scale appearing India, Indonesia, Bangladesh and Vietnam (Altieri, 2010). The expansion may be associated with the enlargement of the agricultural area, advances in biotechnology (that promote significant development in obtaining new varieties of plants), increases of the number of products in the industry and the food quality improvement.

The fast expansion of agribusiness besides the use of finite environmental resources has mobilized various segments of society to adapt the environmental management (Pelizer, Pontieri, & Moraes, 2007). It is estimated that the annual consumption of food in the world is 375 million tons and most of them are vegetables. However, considering only plant foods, about 4 million tons are discarded, generating a significant waste. One-third of the total world population that lives in developed countries consume about 85% of total resources produced in the world (Polaquini, Marcondes, & Rock, 2010).

Despite the increase in productivity, it is estimated that about 20-30% of the harvest of grain, fruit and vegetables in Brazil are wasted in the path between the crop and the consumer, requiring improvements of the system as a whole (Leistritz, Hodur, Senechal, Stowers, McCalla, & Saffron, 2007). Face of this reality, the search for rational utilization of

agricultural production is one of the major challenges for humanity. In many regions the mechanization of agriculture has provided major development creating facilities for subsistence and opening frontiers for intense agribusiness exploration.

However the issue of food production imposes severe demands on unexplored areas, often creating odds with conservationists. In spite of this, the use of appropriate technologies for soil management and improvement in procedures for agricultural production, transportation and storage of food can reduce environmental stress and improve the quality of population’s life (Polaquini et al., 2010).

Agricultural waste

The term "residue" ordinarily is associated with the waste material and devoid of economic value. However, it is known that the majority of solid waste from the exploration of silviculture and agriculture are considered as they include economic value added, allowing its use because it can be converted into raw material for other processes (Silva, Bittar, Serra, & Junior, 2011).

The accumulation of waste by non-use or improper disposal generates environmental damage and loss of resources, contributing significantly to the problem of recycling and conservation of biomass (Pires et al., 2004). Scientific researches show that since 1980 the global environmental problems grow, as the destruction of the ozone layer, the greenhouse effect and the loss of biodiversity, in addition to local impacts from the generation of liquid and solid waste.

These problems demanded a new discussion of the sustainable development model, hitherto inefficient because the generation of waste is associated with the misuse of inputs, losses between production and consumption as well as the materials, throughout the industrial chain (Leistritz, 2007). These waste products can be used as raw material or energy source, given its renewable nature, present great abundance. Biomass is an important source of energy for humanity, whose challenge is to find solutions to use it more efficiently (Alexandriano, Faria, Souza, & Peralta, 2007).

Biomass residues derived from the extraction process increases the potential polluter, such as pollution of soil and water bodies due to improper disposal. On the other hand, the waste management of companies increases the costs associated with handling, transport and disposal of the product generated.

Special attention has been focused on minimizing or reuse of waste as well as proposing new uses for agricultural products and by-products to replace non-renewable resources (Leistritz, 2007). Although they have no apparent economic value, much of generated waste throughout the supply chain can become an important source for the production of new inputs. In these circumstances cease to be problem and begin to generate profit, being converted into raw material for various other processes, reducing the price and demand of the main product (Pires et al. 2004).

In this sense, the development and implementation of sustainable processes capable of converting residual biomass into products with higher added value are indispensable. The processes for full utilization of waste are assuming fundamental role in sustainable agriculture, since they can positively contribute to the minimization of environmental

pollution, besides allowing the economic valuation of these residues (Menezes, Druzian, Padilla, & Souza, 2012) and generate less environmental impact (Rosa, Filho, Figueiredo, Morais, Santaella, & Leitão, 2011).

The use of residues has been studied with the intention of optimizing the production cycle of the agro-industrial chain with minimal waste, exploiting the physical-chemical properties of the residue to produce organic compounds, use of mineral inputs, soil improvement and others (Menegale, Leão, Filho, & Menegale, 2012).

Utilization of the waste

In general, the agro-industrial residues, mainly vegetables, such as fruits, oil seeds, stems, leaves and twigs exhibit diverse chemical composition, which provide opportunities for adding value (Rosa et al., 2011). Depending on the nature of the waste, it can be applied in the field of bioremediation of soils, heavy metals biosorption, as a substrate for double containment, and laboratory animals, among others. The waste build-up is due in large part to the difficulty of being digested and quality products of chemical or enzymatic digestion. However, the use of new technologies can lead products with higher digestibility and adding nutritional value by its use as a renewable carbon source for fermentation processes in large scale allowing the generation of products intended for human consumption and animal feed as well as production of enzymes, organic acids and other (Okonko, Ogunnusi, Aloysius, Adejoye, & Adewale, 2009; Rosa et al. 2011).

Some waste known as bagasse are generated in industries considered waste, basically consist of organic polymers, carbohydrates with low nutritional value. These polymers have potential application in consumption and supplementation in food after physical treatment, chemical and metabolic. In gross condition may serve as a source for fertilizer or animal feed, mainly because it contains a high concentration of carbohydrates (Jerônimo, 2012).

Among the waste, may be exemplified the citrus bagasse which have humidity exceeding 80%, that hinders the transport, storage and application as animal feed. Another option for the implementation of orange waste occurs as a substrate for the cultivation of *Pleurotus ostreatus*, a kind of edible mushroom, popularly called shimeji (Bonatti, Karnopp, Soares, & Furlan 2004).

The biodegradation of these materials has been the subject of many studies, therefore, represents an important step in the carbon cycle in nature. The pre-treatment of lignocellulosic materials with filamentous fungi has been suggested as a way to facilitate subsequent processes of conversion of cellulose into fermentable sugars for bioconversion to ethanol, butanol and other products with commercial interests. The application of the fungi that degrade lignocellulosic materials has also been described in the field of animal nutrition. In this case, the pre-biological treatment is used to increase the digestibility of agro-industrial wastes used as feed (Aguiar and Ferraz, 2011).

Another application of agro-industrial waste is its use as bioadsorventes for the treatment of effluents contaminated with pharmaceutical substances. When compared to other methods, it is shown as a promising proposal, efficient, economically viable and ecologically sustainable (Bila & Dezotti, 2007).

There is a tendency for reasonable use of new technologies, mainly focused on recycling as well as efficient utilization of organic wastes which can be metabolized by various microorganisms (Mazzucotelli, Ponce, Kotlar, & Moreira, 2013).

Particularly, the bioconversion of agricultural residues and food industry has received more attention, because these sectors produce large amounts of waste, both liquid and solid. The solid residue can be utilized more efficiently by the process of solid-state fermentation due to microbial growth and synthesis of several compounds of industrial interest (Pinto, Brito, Andrade, Fraga & Teixeira, 2005).

When this method is applied for the purpose of producing food, it can be obtain a product with better organoleptic and chemical characteristics as well as improved digestibility of the product after fermentation due to degradation of the fibers. Products obtained from the fermentation have an important role in food production, such as flavourings, preservatives and antioxidants (Morales, 2012).

SOLID-STATE FERMENTATION

Solid-state fermentation (SSF) is defined as any fermentation that happens in the complete or partial absence of free water, which occurs the growth of microorganisms on solid materials. However, the moisture necessary for its development is chemically linked to the matter, differently from Submerged Fermentation (SmF) (Hasseltine, 1977; Cannel & Moo-Young, 1980).

Compared to SmF, most commonly used in industrial processes, SSF has several advantages such as: does not require aseptic conditions, allows the employment of a very large variety of raw materials as substrate, requires lower investment and energy, generates less waste even with a potential to process higher volumes in smaller spaces as well as conditions require simpler processing (Sato & Sudo, 1999; Gowthaman, Krishna & Moo-Young, 2001; Durand & Chereau, 1988; Durand, 2003).

Some disadvantages may be found in SSF as the difficulty of mixing, of uniformity of temperature and humidity around the substrate and the detection of microbial growth when compared to SmF (Durand & Chereau, 1988, Sato & Sudo, 1999; Holker, Holfer & Lenz, 2004; Morales; Alcarde & Angelis, 2013). The wider application areas of the SSF are on fermented foods, production of enzymes, and organic acids (Krishna, 2005). Nowadays, the SSF to be attracting attention in recent times due to the possibility of using raw materials of agro-industrial residues with low cost, its application is detected since 2600 BC and bread, cheese and alcoholic beverages appear as some of the older applications (Krishna, 2005; Sato & Sudo, 1999; Durand, 2003).

Although of the ancient use, the most significant application of SSF was developed in the twentieth century when obtained economic significance utilization by the production of enzymes and organic acids (e.g. gluconic acid and citric acid) derivatives from SSF by fungi, as well as production of penicillin during World War II. When the SSF process is under control it is possible to check the production of substances such as mycotoxins (e.g. aflatoxin and ochratoxin) beyond the enrichment of proteins in the production of food. After such discoveries, several studies have been developed using large number of substrates, mainly agro-industrial residues (e.g. pulp, bran, bagasse, lignocellulosic materials, etc.) and

microorganisms in SSF technology (Aidoo, Hendry & Wood, 1982; Pandey, 2003; Pinto et al, 2005; Morales, 2012).

The most common microorganisms used are bacteria and fungi, and filamentous fungi are the most important because they present natural adaptation to the substrate, promoting a faster growth (Raimbault, 1998; Mitchell & Lonsane, 1990). The fact that grow with minimal substrate even at high osmotic pressure and the efficient production of enzyme promotes hydrolysis of the polymers directing the metabolism of monomers to produce a variety of compounds with industrial interest such as flavours, organic acids, antibiotics, pesticides and others, each one produced on a specific substrate and microorganism (Krishna, 2005; Raimbault, 1998).

Factors that influence the SSF

Moisture and water activity

Moisture is the water content in the substrate, referring to surface moisture (easily evaporated) and adsorbed moisture (combined chemically with the substrate) (IAL, 2008).

The optimal level of moisture is essential for the development of SSF stand between 20 and 70% for filamentous fungi and above 70% for bacteria, although variation occurs between 30 and 85%. Low levels of moisture may reduce the transfer of nutrients, microbial growth, as well as stability and activity of enzymes. In contrast, very high levels of moisture promote agglomeration of the substrate hampering the aeration means as well as microbial growth (Lonsane, Ghildyal, Budiatman & Krishnaiah, 1985; Moo-Young et al. 1983; Gowthaman et al. 2001; Pandey, Soccol & Mitchell, 2000; Raimbault, 1998).

Thus, maintaining optimal humidity is directly related to the growth kinetics of the microorganism and the physicochemical properties of the substrate interfering with the performance of SSF (Lonsane, Saucedo-Castaneda, Raimbualt, Roussos, Viniegra-Gonzalez, Ghildyal, Ramakrishna & Krishnaiah, 1992; Ramesh & Lonsane, 1990).

Despite the importance related to moisture in the fermentation substrate, the fundamental criterion related to the water in the SSF is the Water Activity (Aw). Aw is a thermodynamic parameter, which represents the availability of water in a solid substrate as defined by the ratio of the vapor pressure of water in the substrate (p) and the vapor pressure of pure water (p0) at the same temperature (e.g. Aw = p / p 0) (Gowthaman et al. 2001; Raimbault, 1998).

pH

The pH is an important parameter for any procedure involving microbial aerobic and anaerobic metabolism, since each microorganism has an optimal pH range for its growth and activity (Krishna, 2005).

Filamentous fungi can grow in the pH range between 2 and 9, however, the best is between 3.8 and 6.0. Yeasts produce better results at pH 4.0 and 5.0, but can grow between 2.5 and 8.5. Bacteria have the optimum range for growth between 6.5 and 7.5, but can grow between 4.5 and 9.0. Thus, the ability of fungi to grow in highly acidic environments may be used to prevent bacterial contamination in the process (Krishna, 2005; Gowthaman et al. 2001).

Temperature

Probably the temperature is the most important extrinsic factor in SSF. The microbiological growth as well as production of enzymes and metabolites can be affected by the sensitivity of the microorganism to the temperature (Yadav, 1988). Its significance in the process lies in important factors of biological process such as protein denaturation, enzyme inhibition, metabolite productivity and even cell death (Pandey, Soccol & Rodriguez-Leon, 2001).

Precisely this factor appears to be the most difficult to control in SSF due to variations of conduction and convection of the equipment as well as the substrate, beyond the loss of moisture caused by the evaporation from the heating and aeration during fermentation (Trilli, 1986; Auria, Morales, Villegas & Revah, 1993; Raimbault, 1998).

Aeration and Homogenization

Aeration and homogenization are other important factors for SSF, being directly linked to oxygenation in addition of heat and mass transfer in heterogeneous systems. Aeration provides the oxygen supply needed for aerobic processes, in the elimination of carbon dioxide and other volatiles as well as heat dissipation system benefiting microbial growth (Pandey et al. 2001; Lonsane et al. 1992). Several characteristics affect the efficiency of O_2 transfer, such as the pressure and airflow, porosity and thickness of the substrate moist and settings of used fermenters (Lonsane et al., 1992).

The homogeneity of the substrate depends on the processing ensuring equity concentration, humidity, temperature and oxygenation, despite being the factor that presents greater difficulty in SSF, especially in fermenters tray. Some other adversities known by homogenization in SSF are generated by the compression of the substrate (which decreases the gas exchange) and revolving which interfere with the contact between the microorganism and the substrate besides of rupture the fungus micelial filamentary due to shear forces of the system (Trilli, 1986; Banks, 1984; Lonsane et al., 1992; Viesturs, Steinkraus, Milk, Berzines & Tengerdy, 1987; Hesseltine, 1977; Silman, 1980).

Microbial enzymes

Enzyme production is a rapidly growing field reaching about 2 billion dollars in sales annually. This volume grows 4-5% a year on average. The main markets of the enzyme industry cover the areas of technology, food and animal feed. Phytase enzymes lead the area of animal feed, while in technology area, enzymes for detergents and pulping of wood are the most application. In the food industry, the market leaders are proteases and amylases (BCC, 2004).

In addition to these well-established market areas, the microbial enzymes have been gaining market areas of biotransformation involving organic solvents and bioactive compounds (Gowthaman et al., 2001; Pandey et al., 2000). Even with the advantages presented by the SSF for the production and purification of enzymes, the most widely used remains the SmF, usually using modified organisms. However, the SSF has shown significant advantageous results when compared to SmF using the same microorganism, as the stability of extracellular enzymes and low levels of catabolic repression (Viniegra-Gonzalez, Favela-Torres, Aguilar, Romero-Gomez, Diaz & Gordinez Augur, 2003; Krishna, 2005).

A large number of microorganisms used in the industry including bacteria, yeasts and molds produce several kinds of enzymes but this production is mainly derived from filamentous fungi with the exception of alkaline proteases (Babu & Satyanarayana, 1996).

To improve the industrial production, agro-industrial residues are considered the finest substrates for the production of enzymes because they have required physical-chemical characteristics for microbial growth at low cost (Gowthaman et al. 2001).

Organic acids

Organic acids are among the most commonly used ingredients in the food and beverage industry, primarily as acidulant. Citric acid and lactic acid have been produced by SSF and recently include fumaric, oxalic and gallic acid. In addition of acidulant citric acid ($C_6H_80_7$) is also widely used in industry as antioxidant and chelating agent in foods, beverages, pharmaceuticals, cosmetics and other products. Various filamentous fungi can produce citric acid, but recently mutants of *Aspergillus niger* has been used commercially in this production, especially in the agro-industry by-products like coffee husk, cassava bagasse, wheat and rice bran, etc. (Pandey et al. 2001, Sato & Sudo, 1999).

Lactic acid ($C_3H_60_3$), another important organic acid is obtained about 50% by chemical synthesis and 50% by fermentation. An important application of lactic acid is in the production of biodegradable polymers and coatings in addition to resins, herbicides and pesticides (Pandey et al. 2001). The lactic acid produced by the SSF may be obtained by the use of fungi and bacteria, especially by *Rhizopus* and *Lactobacillus*sp. It can also be produced by fermentation of agro-industrial by-products like sugarcane bagasse, waste of carrots, as well as sorghum and cassava (Pandey et al., 2000, 2001).

Antibiotics

Antibiotics are organic compounds of low molecular mass produced by some microorganisms, usually under stress conditions, which have activity against other microorganisms when present in low concentrations (Demain, 1999).

Some examples of known antibiotics are penicillin (produced by fungi of the genus *Penicillium*), cyclosporine (which has antifungal, anti-parasitic, anti-inflammatory and immunosuppressive activities), the cephamycin (broad spectrum antibiotic), tetracycline (also broad spectrum which inhibit Gram-positive and negative bacteria and others microorganisms) among others antibiotics (Robinson et al., 2001; Balakrishnan & Pandey, 1996; Ohno, Ano & Shoda, 1992, 1993; Ohno, Takashi & Shoda, 1995).

About 12,000 antibiotics were known a few years ago, being about 55% produced by actinomycetes, 12% by bacteria and 22% by filamentous fungi (Demain, 1999).

Mycotoxins

Substances produced by fungi as a secondary metabolite has received special attention in recent years. Most of these toxins cause severe health problems in animals and humans. Aflatoxins are the most popular because they are the ones that present the largest biological potential. Mycotoxins present great danger and can accidentally appear in SSF, either by the presence of the toxin in the raw material or the production from accidental contamination (Krishna, 2005; Gonzales & Tomasini, 1996).

Biofuels

The most important biofuel produced by SSF is bioethanol. Ethanol is the most widely used biofuel nowadays being produced mainly by SmF in a system similar to the production of wine and beer with subsequent distillation. However, the bioconversion of cellulose and hemicellulose has been studied extensively for the development of lower cost technologies using mainly residues from agro-industry (Chandrakant & Bisaria, 1998; Morales et al., 2013).

Biomass

The attainment of biomass by SSF is one application that presents a very promising situation and its application considerably increases nowadays. The quantification of microbial biomass during the fermentation is crucial for the assessment of microbial growth and its calculation essential for growth kinetic studies of SSF leading to recognise the end of the process. Another important metabolic activity of the biomass estimation is the production of extracellular enzymes, because there is a relationship between growth and hydrolytic enzymes such as amylase, cellulase and pectinase as well as organic acids (Raimbault, 1998; Roussos, Raimbault, Viniegra-Gonzalez, Saucedo-Castaneda & Lonsane, 1982).

Another way of measuring the biomass is by determination of specific components such as proteins, nucleic acids and ergosterol (Scotti, Vergoignan, Feron & Durand, 2001; Sakurai, Lee & Shiota, 1977; Morales, 2012). A parameter that has proved suitable for biomass estimation in an aerobic SSF is given by the ratio of consumed oxygen and release carbon dioxide, but the biomass estimate is more suitable for processes where the major product is the biomass itself and for the production of secondary metabolites these products are the best indicators of growth and expression of the activities (Krishna, 2005).

Nutritional factors

All substrates with potential for utility in SSF must provide minimum nutrients to ensure the efficiency of the fermentation, such as carbohydrates, proteins, minerals and other cofactors (Krishna, 2005; Melo, Rangel, Barreto, Ibañez-Rojas & Moreira, 2007).

Carbohydrates are the main energy sources for the maintenance of the physiological processes of the microorganism, may be in the form of monosaccharide such as glucose or more complex such as cellulose and starch. Other types of carbohydrates can also be used to produce energy by microorganisms (e.g., sucrose, lactose, glycerol, etc.) as well as lipids and proteins can represent this function in the absence of carbohydrates (Evangelista, 2000; Krishna, 2005).

However, the main function of the proteins is structural or plastic and is associated with the growth, repair and development of microorganisms. Related to these two factors, the ratio of carbon (from carbohydrates) and nitrogen (from protein) is essential for optimal development of fermentation being this ratio is typically in the range of 16:1 (C: N). The function performed by vitamins and minerals is to regulate the metabolic and increase sporulation of the fungus (Evangelista, 2000; Krishna, 2005; Larroche, 1996). Acting on appropriate substrates, the cell biomass from the SSF has a range of 40-50% carbon, 30-50% oxygen, 6-8% hydrogen and 3-12% nitrogen (Pandey et al., 2001).

Morales (2012) obtained results that show the efficiency of the SSF in the improvement of protein composition by analysing the amino acid profile, especially in the considered

essential, before and after the fermentation of cassava leaves with *Rhizopus oligosporus*. This material is a high protein content residue, generated after the harvest of cassava and present a potential for production about 2250 kg per hectare in dry weight without competing with tubers (main product explored in cassava culture) (Cereda, 2001).

These features and the results obtained by SSF show the potential application of the biotransformation of edible fungi in agro-industrial residues for obtaining products of commercial interest.

CONCLUSIONS

Agricultural productivity reaches record highs with increases expectation. Due this, tons of residues are generating. Features about these wastes allow the use as inputs with low costs as well as new products. The ability to use these residues can attain high amounts although not fully exploited. The solid-state fermentation is one option to the great use of agro-industrial residues by the capacity of the application the waste as an ideal substrate to this operation. The easy adaptation mainly by fungi to ferment this kind of substrate can ensure the obtainment of large quantities of commercial product by lower costs and other facilities as the concentration of biomass and enzymes. Thus, obtaining new inputs, products and chemicals from agro-industrial residues by solid-state fermentation can be another tool in promoting the sustainable use of natural resources.

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